Immunotherapy in the battle against cancer: David versus Goliath?

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**Immunotherapy in the battle against cancer:
David versus Goliath ?**

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Immunotherapy in the battle against cancer: David versus Goliath ?

PROEFSCHRIFT

Ter verkrijging van de graad van doctor aan de Universiteit Maastricht, op gezag van de Rector Magnificus, Prof. mr. G.P.M.F. Mols, ingevolge het besluit van het College van Decanen, in het openbaar te verdedigen op vrijdag 9 maart 2012 om 10:00 uur.

Door

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Voor Pap en mam

1 Samuel, chapter 17

Saul and the Israelites are facing the Philistines at the Valley of Elah. Twice a day for forty days, the giant Goliath, the champion of the Philistines, comes out between the lines and challenges the Israelites to send out a champion of their own to decide the outcome in single combat. However, Saul and all the other Israelites are afraid of him. David, who brought food for his elder brothers is also present at the front lines. Told that Saul has promised to reward any man who defeats Goliath, David accepts the challenge. Saul reluctantly agrees and offers his armor, which David declines, taking only his sling and five stones chosen in a brook.

David and Goliath confront each other, Goliath with his armor and shield, David with his staff and sling. "The Philistine cursed David by his gods," but David replies: "This day the Lord will deliver you into my hand, I will strike you down, cut off your head, and I will give the dead bodies of the host of the Philistines this day to the birds of the air and to the wild beasts of the earth; that all the earth may know that there is a God in Israel, and that all this assembly may know that God saves not with sword and spear; for the battle is God's, and he will give you into our hand."

David hurls a stone from his sling with all his might, and hits Goliath in the center of his forehead. The Philistine falls on his face to the ground; David takes his sword and cuts off his head. The Philistines flee and are pursued by the Israelites "as far as Gath and the gates of Ekron." David puts the armor of Goliath in his own tent and takes the head to Jerusalem, and Saul sends Abner to bring David to him. The king asks whose son he is, and David answers, "I am the son of your servant Jesse the Bethlehemite."

1 Samuel, hoofdstuk 17

Saul en de Israëlieten zijn in gevecht met de filistijnen in de Vallei van Elah. Tweemaal per dag, voor veertig dagen staat de reus Goliath, de sterkste man van de filistijnen tussen de linies en daagt de Israëlieten uit voor een man tegen man gevecht tegen hun sterkste man. Saul en alle Israëlieten zijn bang voor Goliath. David, die zijn oudere broers van voedsel voorziet is ook aanwezig bij de front linies. Zijn broers vertellen hem dat degene die Goliath verslaat door Saul rijkelijk beloond zal worden. David wil deze strijd aan gaan en weet Saul ervan te overtuigen dat hij de Goliath kan verslaan. Saul stemt uiteindelijk in en biedt David zijn zwaard aan. David slaat dit aanbod echter af.

David en Goliath komen tegenover elkaar te staan. Goliath met schild en zwaard en David alleen met zijn herdersstaf en zijn slinger. De filistijnen vervloeken David en zijn god, maar David antwoordt: "Vandaag zal God jou aan mij uitleveren, ik zal je verslaan, je hoofd eraf hakken en ik zal de dode lichamen van de filistijnen aan de vogels van de lucht en de beesten van de aarden geven; zodat de wereld zal weten dat er een God bestaat, en dat iedereen zal weten dat God niet redt met zwaard en speer, voor dit is Gods strijd."

David neemt een steen en werpt deze met zijn slinger uit al zijn kracht richting Goliath. De steen raakt Goliath in het voorhoofd. Goliath valt voorover met zijn gezicht op de grond. David neemt zijn zwaard en hakt zijn hoofd eraf. De filistijnen vluchten, achterna gezeten door de Israëlieten verder dan Gath en de poorten van Ekron. David neemt het hoofd van Goliath naar Jeruzalem en Saul stuurt Abner om David te halen. Saul vraagt wiens zoon hij is en David antwoordt: "Ik ben David de zoon van Jesse uit Bethlehem."

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List of abbreviations

Abbreviations

Ab	Antibody
ADC	Antibody-dependent complement activation
ADCC	Antibody-dependent cellular cytotoxicity
AML	Acute myeloid leukemia
APC	Antigen presenting cell
BCG	Bacilli Calmette-Guérin
cAMP	Cyclic Adenosine monophosphate
CB	Cord blood
CD	Cluster of differentiation
CEA	Carcinoembryonic antigen
CHO	Chinese hamster ovary
CML	Chronic myeloid leukemia
COX	Cyclooxygenase
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen-4
DC	Dendritic cell
DNA	Desoxyribonucleic acid
DNAM-1	DNAX accessory molecule-1
FDA	Food and drug administration
FI-DC	FMKp/IFN- γ DC, differentiated with IL-13
FMKP	Membrane fraction of Klebsiella pneumoniae
Gal	Galactose
GalNAC	N-acetylgalactosamine
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GvHD	Graft-versus-Host disease
GVT	Graft-versus-tumor
H	Hour
hDLL	Human Delta-like ligand

HLA	Human leukocyte antigen
iDC	immature Dendritic cell
IFN	Interferon
IL	Interleukin
KIR	Killer immunoglobulin-like receptor
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MDSC	Myeloid-derived suppressor cell
MFI	Mean fluorescence intensities
MICA/B	MHC class I-related chain A/B
MUC1	Mucin 1
NCR	Natural cytotoxicity receptor
NK cell	Natural killer cell
NK-22 cell	Natural killer-22 cell
NKT cell	Natural killer T cell
PAMP	Pathogen-associated molecular pattern
PGE	Prostaglandin
Poly (I:C)	Polyribocytidylic acid
PRR	Pattern recognition receptor
SCT	Stem cell transplantation
SEB	Staphylococcal Enterotoxin B
SEM	Standard error of the mean
Ser	Serine
TAA	Tumor-associated antigen
T _{CM} cell	Central memory T cells
TCR	T cell receptor
T _{EM} cell	Effector memory T cells
T _H cell	Helper T cell
Thr	Threonine
TIL	Tumor infiltrating lymphocytes
TLR	Toll-like receptor

TNF	Tumor necrosis factor
TP-DC	TNF- α /PGE2 Dc, differentiated with IL-13
Treg	Regulatory T cell
WT-1	Wilms' tumor 1

Chapter

1

**General introduction:
Immunotherapy in the battle against
cancer: David versus Goliath?**

Cancer and immunotherapy

In the story of David and Goliath, which was written centuries ago, we were taught that a battle can only be won by intelligence and not by pure strength. Only when targeting the enemy's weak spot David was able to conquer Goliath. In analogy to this story, also in the battle against cancer pure strength is not enough. Where several destructive anticancer therapies have failed to cure all cancer patients, immunotherapy might be a more clever strategy, since it specifically targets cancer cells aiming at their weakness.

With approximately 25% of all deaths, cancer is the leading cause of mortality in Western countries. Since the early nineties the mortality rates of cancer have decreased, which is due to early detection and treatment of pre-malignant and malignant lesions. However, the lifetime probability to develop cancer is with 44% for men and 38% for women, still high (1). Most cancer patients are treated with a combination of surgery, chemotherapy and radiation and advances in these therapies have improved cancer survival. At this moment approximately 50% of all cancer patients survive (2), which is not only due to improvements of conventional therapies, but also by the use of new treatment options, including immunotherapy. Favorable results of immunotherapy that were reported over the last decades, hold promise for the future.

The immune system

In steady state conditions, when invading pathogens or malignant cell transformation perturbs normal tissue homeostasis, tissue responses result in innate immune cell infiltration and initiate an acute inflammatory response (Figure 1). Inflammatory mediators produced by early infiltrating macrophages, neutrophils and mast cells recruit other innate and adaptive immune cells into the damaged tissue. Additionally, these immuno-active mediators induce tissue and vascular responses that increase tissue perfusion and repair. Tissue resident antigen presenting cells (APCs), like dendritic cells (DCs), engulf foreign or altered-self antigens and migrate into the lymph nodes to present these antigens to cells of the adaptive immune system (3, 4). Recently, it has been demonstrated that natural killer (NK) cells bidirectionally interact with DCs at the interface of innate and adaptive immunity (5-7). Additionally, NK cells are efficient innate effector

cells that can rapidly eradicate infected or malignantly transformed cells by a specialized machinery of activating and inhibitory receptors on the NK cell surface. NK cells can do this without the need of memory or antigen specificity (8).

The innate immune system orchestrates the induction of adaptive immune responses. Efficient activation of adaptive immune cells, T and B cells, is only established upon encounter with APCs. T and B cells are committed to recognize specific antigens by generation of antigen receptors by somatic recombination. Upon antigen recognition clonal expansion of T and B cells is needed to induce an effective immune response (9, 10). Therefore, compared to the innate system, the adaptive immune system is more specialized, but also slower (days compared to hours) in mounting immune responses. However, due to induction of memory cells, the adaptive immune response is initiated more rapidly upon a second encounter with the same antigen (11) (Figure 1).

Eventually, the activation of both the innate and adaptive immune system is needed to conquer infection and cancer. After elimination of pathogens or pathological cells, the immune response is suppressed to reconstitute normal tissue homeostasis. However, if normal tissue homeostasis is not reached a state of chronic inflammation is induced. During this state interactions between immune cells are disturbed, which attributes to cancer formation (12, 13).

Figure 1. The immune response.

The mammalian immune system has two overarching divisions, the innate and the adaptive. The innate immune system includes, among other components, various phagocytes that can ingest and destroy pathogens or apoptotic cell particles. These cells also activate the inflammatory response by secretion of chemokines and cytokines, thereby recruiting and activating other innate immune cells. One of these immune cells migrating from the blood into the inflamed tissue is the NK cell, which is an efficient killer of both infected as well as malignantly transformed cells. For activation of the adaptive immune system, DCs have to migrate into the lymph node, where they can present their antigen to T cells, thereby activating these cells. This T cell activation is enhanced by NK-DC crosstalk, cells which are both at the interface of the innate and adaptive immune system. After antigen recognition, antigen-specific T cells start to proliferate and both effector and memory T cells are formed. The effector T cells migrate into the inflamed tissue to eliminate pathological cells. Memory T cells are of importance to promptly eliminate invaders upon a second inflammatory response. B cells differentiate by the same pattern into effector and memory B cells. Effector B cells are plasma cells that secrete antibodies, which can bind pathological cells and induce antibody-dependent complement activation or antibody-dependent cellular cytotoxicity (ADCC).

The paradox of the immune system and cancer

The first observation of the involvement of the immune system in cancer was described in 1863 by Rudolph Virchow, who identified immune cells residing in tumor tissue (14). Later, it was shown that clinical prognosis is associated with the type of immune cells that infiltrate this malignant tissue (15, 16).

A medium and high cytotoxic activity of peripheral blood lymphocytes is associated with reduced cancer risk, whereas low activity is associated with an increased risk suggesting a role for natural immunological host defense mechanisms against cancer (17). This is supported by the observation that immuno-deficient mice as well as organ transplant patients treated with immune-suppressive therapy are more prone to develop cancer (18-22).

Not all activities of the immune system are protective for cancer development. Cancer patients often harbor T cells that are functionally impaired (23), contain NK cells that express lower numbers of activating receptors (24) and accumulate chronically activated myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Treg) (25, 26). Increasing evidence suggests that chronic inflammation is associated with tumor development or tumor growth and about 15% of all malignancies can be attributed to infections (27, 28). Examples of infection and inflammation that lead to an increased risk in cancer development are: *Helicobacter pylori* infection, causing stomach cancer (29); human papillomavirus infection, which is associated with cervical cancer development (30); chronic pancreatitis leading to pancreatic cancer (29); and inflammatory bowel disease, which predisposes for the development of colon cancer (31, 32). This can be explained by the inflammation-induced production of high concentrations of reactive oxygen and nitrogen species that have potential DNA-damaging effects. In an inflammatory environment, characterized by cell renewal and proliferation, these effects on the DNA can be detrimental and result in cancer formation (33). However, not every infected individual develops cancer, indicating that cancer development is a multifactorial process. Possible factors responsible for cancer development are genetic predisposition (mutations or polymorphisms), toxic environmental factors, and the immune status of the individual (22, 34).

In apparent paradox to the data on predisposition of cancer development in immuno-compromised individuals, long-term treatment with aspirin or cyclooxygenase (COX) inhibitors, which also suppress the immune response, reduces the risk of tumor development (35-37). Both anti-inflammatory drugs inhibit the production or function of prostaglandins (PGE) and vasoactive molecules that are involved in immune cell activation. Since inadequately high amounts of PGE are produced during chronic inflammation, these drugs suppress chronic inflammation and ultimately tumor formation (38).

In conclusion, next to genetic predisposition and environmental factors, tumor development depends on a delicate equilibrium of inflammation-related tumor induction, tumor immune surveillance and tumor escape (39). On the one hand, chronic inflammation induces tumor formation and established tumors actively secrete immuno-active molecules, that potentiate neoplastic progression (27). On the other hand, the immune system provides us with a well-orchestrated machinery of tumor surveillance, which is effective in recognition and rejection of malignant cells, also known as immuno-editing (Figure 2) (40). This function of the immune system opens new opportunities for cancer immunotherapy.

Figure 2. The paradox of cancer and the immune system.

Immune surveillance is needed for tumor elimination, but is also responsible for the recruitment of immune cells. Immune cells produce pro-inflammatory mediators, creating a tumor-promoting environment that leads to tumor progression. Immunotherapy aims at enhancing immune surveillance and thereby tumor elimination.

Cancer immunotherapy

The main goal of cancer immunotherapy is to augment antitumor immunity and inhibit immune regulatory responses that lead to tumor tolerance. Different immunotherapeutic strategies have been classified as either active- or passive therapy (Figure 3). Passive immunotherapy employs immune components created outside of the body that provide specific immunity after administration. In contrast, active immunotherapy stimulates the body’s own immune system to fight cancer. These cancer immunotherapeutic strategies can either be aspecific or specific, thereby enhancing general immune responses or antigen-specific responses, respectively. Its specificity is the major advantage of immunotherapy over conventional anticancer therapy (e.g. chemotherapy and radiotherapy), rendering only cancer cells to be targeted without harming normal tissue. As a consequence, treatment related morbidity and mortality are reduced. Though the subdivision between passive and active immunotherapy seems very strict, there is a substantial overlap between the two different therapeutic strategies.

Figure 3. Cancer immunotherapy.
Schematic representation of different immunotherapeutic approaches categorized in either passive- or active immunotherapy and further subdivided in specific or aspecific.

Passive immunotherapy

The main effectors of passive immunotherapy are antibodies that recognize antigens expressed by tumor cells. Over the last decade also antibodies targeting immune cells have been developed. These strategies use passive immunotherapeutic components to induce active immune responses. Other forms of passive immunization are adoptive T cell transfer of either antigen-specific T cells or Pre- T cells, adoptive NK cell transfer and stem cell transplantation (SCT).

Antibody therapy

Antibodies (Ab) produced by B cells have the capacity to bind immunogenic tumor cell surface molecules and mediate tumor cell lysis by complement activation (ADC) or induction of antibody-dependent cell-mediated cytotoxicity (ADCC) (Figure 4). Additionally, these Abs can bind receptors inducing steric inhibition or neutralization of cytokines, thereby interfering with the tumor cell cycle or providing pro-apoptotic signals. Furthermore, Abs can complex with free antigens, augmenting engulfment, processing and presentation of these antigens by APCs.

Transfer of monoclonal antibodies (mAbs) is the most successful form of immunotherapy and over the last years numerous mAb therapies have been developed (40-44). Favorably, these mAbs are directed to specific immunogenic epitopes overexpressed by tumor cells, since there is only a negligible chance of autoimmune reactions (45). Such tumor-associated antigens (TAA) often display differences in their glycosylation pattern or represent alternative splice variants (46). To enhance the therapeutic effect of mAbs, they can be linked to radionuclides or toxins (47). The most well-known mAb used in clinical practice is the CD20-binding antibody Rituximab. Though successful in anticancer therapy, CD20 is not a TAA since it is not only expressed in many B cell lymphoma but also by healthy B cells. Therefore, Rituximab induces ADCC of malignant as well as non-malignant B cells (48). It is added to the standard chemotherapy protocol of non-Hodgkin's lymphoma (cyclophosphamide, doxorubicin, vincristine and prednisone), because the percentage of patients reaching complete remission increased significantly (48, 49). Another mAb widely applied in cancer treatment is Trastuzumab, which binds the Her-2/Neu receptor often expressed by breast cancer cells (50).

Figure 4. Induction of antibody-mediated immune responses.

Humoral immune responses can be established by passive antibody transfer, which can induce antitumor responses by ADC and ADCC. Bispecific antibodies are used to link tumor and effector cells inducing tumor cell lysis. Antibody transfer can also induce active immunization by enhancing antigen presentation by antigen-coupled antibodies that bind to DCs or by activation of macrophages and DCs due to ligation of the CD40 costimulatory receptor. TCR = T cell receptor, NCR = natural cytotoxicity receptor, KIR = Killer immunoglobulin-like receptor.

More advanced immunotherapeutic approaches use passive antibody therapy to induce active immune responses. An example is the use of bispecific antibodies that target tumor antigens as well as immune effector cells (51). This *in vivo* targeting strategy is also used to couple tumor-specific antigens to antibodies that specifically bind APCs, thereby inducing phagocytosis and antigen presentation (52, 53) (Figure 4). More advanced are the new-generation mAbs that have immune-modulating effects, like antibodies blocking cytotoxic T lymphocyte antigen-4 (CTLA-4), which leads to decreased activation of Tregs, thereby enhancing antitumor immunity (54).

In vivo data have shown that the use of mAbs as monotherapy has only a minimal effect on survival, however, in combination with conventional therapies or as a preventive adjuvant-treatment after tumor remission mAb treatment has proven to be much more effective (55).

Adoptive T cell transfer

Adoptive T cell transfer is based on the *ex vivo* identification of autologous or allogeneic T cells that display antitumor activity. These cells are then *ex vivo* expanded and activated before being reinfused. Before reinfusion, the patient's immune system can be effectively manipulated by chemotherapy, radiotherapy or surgery to exert optimal T cell responses (56, 57). T cells, used in adoptive T cell transfer can be obtained from the peripheral blood, the tumor draining lymph nodes or from the tumor itself (tumor infiltrating lymphocytes (TIL)) (Figure 5).

Figure 5. Adoptive T cell therapy.

TILs are isolated, cultured and selected for their specific T cell receptor expression. T cells expressing high avidity T cell receptors recognizing tumor cells are selected and proliferation/activation of these cells is induced. Eventually they are re injected into the cancer patient to induce antitumor responses.

The effectiveness of adoptive T cell transfer was first reported in the context of melanoma, where TILs showed antitumor reactivity. These TILs were expanded *ex vivo* and then re-injected into melanoma patients where they showed effective *in vivo* antitumor activity (58). Although initial clinical response rates were high, antitumor responses were not long lasting, since the median survival of injected TILs was shorter than one week (59). To improve clinical responses, only very pure T cell fractions were used with effective depletion of Tregs (60). Even more important to prevent immune responses against the newly infused T cells, cancer patients were actively lymphodepleted before re-infusion (61). Not only CD8⁺ T cells have been identified to display potent anticancer reactivity (62) but also CD4⁺ T cells have been implicated in this reactivity (63). Adoptive T cell transfer has been proven to induce tumor regression in gastric cancer (64), renal cell cancer (65-67), melanoma (68) and lung cancer, however the effects on overall survival are disappointing and vary the different cancer subtypes.

The induction of tumor regression by T cell transfer in anticancer therapy, has led to the development of genetically altered T cells. Using retroviral vectors, genes encoding T cell receptors (TCRs) can be effectively transduced into lymphocytes to redirect their specificity (69-71).

Another new approach in T cell transfer is the development of *ex vivo* generated T cells from stem cells. In animal models these techniques have been shown feasible and T cell progenitors have been isolated (72). In mice, important signaling pathways for *in vitro* T cell development have been identified, and amongst others Notch and Flt3 signaling are essential signals (73, 74). In humans it has been proven to be more complicated to generate T cells *in vitro* (75, 76). The only successful approaches rely on the use of stromal cell lines (77) or the injection of precursor T cells into humanized mice (78, 79), indicating that a thymic microenvironment is needed for human T cells to develop. It is not certain if fully mature T cells are needed for effective adoptive T cell transfer. For T cell repopulation after immuno-ablative anticancer therapy (like in stem cell transplantation) T cell precursors might also form an attractive option. Many obstacles, however, have to be overcome before such therapies can be applied in cancer patients. For instance, the functionality of stem cell-derived T cells remains to be determined. Additionally, in the context of reinfusion of mature T cells, it is not known whether the *in vitro* culture systems

induce positive and negative selection and thereby potentially generating auto-reactive T cells (80). With the use of T cell progenitors this could be prevented, since the selection process is completed in the patient's own thymus. Nevertheless, it is still unclear whether *in vitro* generated T cell precursors will migrate into the human thymus.

Both, autologous as well as allogeneic T cells can be used for adoptive T cell transfer for cancer. Allogeneic T cells are able to kill tumor cells not only by the recognition of specific tumor antigens but also by other non-self antigens. However, these therapies are often complicated by aggressive graft-versus-host disease (GvHD). Since allogeneic T cells do not distinguish tumor cells from normal tissue cells, new protocols have been developed to deplete alloreactive T cells recognizing healthy tissue (81).

In summary, T cells can be adoptively transferred in cancer patients. Adoptive transfer of antigen-specific T cells is a form of specific, passive immunotherapy, whereas transfer of pre-T cells and allogeneic T cells are considered a form of aspecific, active immunotherapy. Even though antitumor responses have been reported for both forms of adoptive T cell transfer, improvement of these therapeutic strategies is needed to further enhance the clinical effectiveness.

NK cell therapy

NK cells are the most efficient effector cells of the innate immune system and were first described in 1975, when it was shown that natural cytotoxic cells were able to kill tumor cells without being previously sensitized (24, 82). The function of NK cells was eventually described by Kärre et al, who postulated the missing-self hypotheses and proved in mice that MHC class-I deficient lymphoma cells were rejected (83). Later this mechanism was extended with the addition of the induced-self hypothesis (Figure 6) (84). Both hypotheses describe the ability of NK cells to kill target cells. This cytotoxic activity is based on a delicate balance of inhibitory and activating signals. The balance depends on the expression of MHC class I and activating ligands on the target cell as well as on expression of the respective receptors recognizing these ligands by NK cells. The missing-self hypothesis describes the capacity of NK cells to kill target cells due to their altered expression of MHC class I. When a cell becomes virally infected or malignantly transformed, MHC class I expression is downregulated to overcome T cell-mediated tumor

cell lysis, however decreased class I expression makes tumor cells more vulnerable for NK cell kill. Activation of NK cell cytotoxicity is mediated by killer immunoglobulin like receptors (KIRs) expressed on the NK cell surface. Upon engagement of these inhibitory KIRs with MHC class I molecules of a target cell, inhibitory signals are transmitted, prohibiting NK cells to kill the target cell. However, if the inhibitory KIRs cannot bind their MHC class I ligand, they become licensed to kill the target cell (85). Killing of these MHC class I missing target cells can only be initiated after simultaneous detection of activating signals, mostly stress signals, on the surface of tumor cells or ligation of activating KIRs. Expression of numerous activating receptors can also induce NK cell killing even in the presence of MHC class I surface expression, this mechanism is described in the induced-self hypothesis (Figure 6).

Figure 6. NK cell function.

NK cells are activated by a delicate balance of activating and inhibiting signals. *Upper:* by interacting with normal tissue cells that express MHC class I and stress ligands, NK cells do not become activated because the inhibitory signal is stronger than the activating signal. *Middle:* by interacting with tumor cells that upregulated the amount of stress molecules, NK cells become activated because the activating signals overrule the inhibitory signal (induced-self). *Lower:* Interaction with tumor cells that have downregulated their expression of MHC class I, will lead to NK cell activation (missing-self).

To make use of these NK cell characteristics, adoptive NK cell therapies have been introduced. Investigators reasoned that NK cell activity in cancer patients is altered. Therefore, NK cells from patients were harvested and activated *ex vivo* with IL-2 before being reinjected into the patient (86, 87). Although some clinical responses were reported, most autologous NK cell protocols did not induce significant effects. Other lines of research focused on the use of stem cell-derived NK cells in which KIR profiles can be altered or to make use of NK cell lines expressing all KIRs (88, 89), however these techniques have been proven to be difficult and are still under investigation. Additionally allogeneic NK cells are used in anticancer therapy, which will be discussed in more detail in the next paragraph in relation to stem cell transplantation (SCT).

Stem cell transplantation

Hematopoietic stem cell transplantation (SCT) has been suggested to be a form of passive immunotherapy, which has been proven effective in most hematological malignancies and some solid tumors. Since the classification of active and passive immunotherapy sometimes seems a bit arbitrary, SCT has also been classified as active immunotherapy. Both autologous and allogeneic SCT are currently utilized in clinical practice. Autologous transplantation replaces the patient's own bone marrow after intense myeloablative chemotherapy and is therefore considered as a rescue treatment and not a form of immunotherapy.

In allogeneic SCT, antitumor responses are induced by the graft-versus-tumor (GVT) effect exerted by the donor's immune cells and not by the intensive chemo- and or radiotherapy prior to transplantation. Patient and donor are matched for major human leukocyte antigen (HLA) class I and II molecules, however minor mismatches still exist. Due to these minor mismatches and due to the inflammatory response after tumor cell damage induced by the conditioning therapy, alloreactive T cells can exert antitumor responses (90). Hematopoietic stem cells can be derived from bone marrow or cord blood, but the most convenient source is the isolation of granulocyte colony-stimulating factor (G-CSF) mobilized stem cells from the peripheral blood (91). Stem cells can either be obtained from HLA-matched siblings or from unrelated matched donors (volunteers that are registered in a worldwide database). To ablate the immune system, patients are pre-

treated with intense chemo- and/or radiotherapy, thereby reducing the tumor load and more importantly enhancing engraftment due to suppression of the recipient's immune system (91).

Next to the antitumor effect, SCT also causes serious side effects. Alloreactive T cells are not only capable of inducing GVT effects, but can also destruct healthy cells. This process is known as graft-versus-host disease (GvHD) (92). Another side effect of SCT is related to the toxicity of the preconditioning, which makes allogeneic SCT only available for young patients. However, with the development of the new non-myeloablative SCT with reduced toxicity also older patients can be transplanted (93, 94). Additionally, the intense conditioning is responsible for immuno-ablation, which is associated with an increased risk of opportunistic and viral infections, due to neutropenia and slow T cell recovery (95). Currently new techniques are being developed to deplete the transplanted stem cell fraction only of alloreactive T cells that could induce GvHD and not T cells that can fight infection (96).

Figure 7. Effects of alloreactive NK cells in haploidentical stem cell transplantation.

If NK cells express one or more KIRs that cannot bind MHC class I (HLA-C1, C2, or Bw4), they become activated and kill the target cell (*upper and middle*). However if the tumor cell expresses all KIR ligands, NK cells are never able to exert allogeneic activity (*lower*).

Another form of alloreactive SCT is the mismatched haploidentical SCT, where the GVT effect is not only based on T cell alloreactivity, but also on the presence of alloreactive NK cells. Donors are selected on a HLA-KIR mismatch, which means that the recipient lacks one or more of the HLA class I ligands for donor inhibitory KIRs. Such a KIR mismatch will lead to alloreactive NK cell activation (Figure 7). In patients with acute myeloid leukemia (AML) treated with a SCT where donor and recipient displayed a KIR-HLA mismatch, it was shown that there was an increase in survival and reduced relapse rates (97). Notably, SCT that involved a KIR mismatch showed a reduced risk of GvHD development and increased percentage of engraftment. The capacity of alloreactive donor NK cells to kill recipient antigen presenting cells accounted for the decreased development of GvHD (97, 98). Despite the positive effects obtained by initial studies of mismatched haploidentical SCT in AML patients, other studies failed to reproduce these results (99, 100). Discrepancies might be caused by differences in donor selection or by differences in the conditioning regimen (101).

Because of the successes reported using haploidentical stem cells, many studies focus on using allogeneic NK cells in adoptive cell therapy for cancer (102). Adoptive transfer of these cells has been shown to be well tolerated and does not induce GvHD. Especially in patients with AML, haploidentical NK cell therapy has been proven successful (97).

Allogeneic SCT is effective in cancer treatment and is therefore standard treatment in many hematological malignancies. However, toxicity of the conditioning regimen, graft failure, increased risk of infectious disease and development of GvHD form life threatening complications and indicate that optimization of SCT is needed.

Active immunotherapy

Activation of the body's own immune system can be aspecific, activating of overall immune responses or antigen specific. Antigen-specific immune responses can be achieved by administration of antigens or antigen loaded DCs. Aspecific activation is mediated by adjuvants, like cytokines and chemokines.

DC vaccination

Therapeutic DC vaccination strategies aim at activating the cancer patient's immune system to overcome tolerance and to induce effective antitumor immune responses (4).

In this strategy, DCs are obtained from the cancer patient, stimulated *ex vivo*, loaded with tumor antigens and then re-injected into the patient where they have to migrate into the draining lymph nodes. In these lymph nodes they bring about clonal expansion of T cells expressing a specific randomly arranged antigen-specific receptor (103, 104).

For the *ex vivo* generation of DCs, several different DC subsets have been used including monocyte-derived DCs, stem cell-derived DCs, DC cell lines (105) and DCs obtained from dedifferentiated AML cells (106). In most clinical DC vaccination therapies, however, monocyte derived DCs are administered (107). In these vaccination therapies, monocytes are induced to differentiate into immature DCs (iDC) by cocktails containing GM-CSF in combination with either IL-4, IL-13 or IL-15 (107). The capacity of DCs to present antigen, express co-stimulatory molecules, home into the draining lymph nodes and to secrete cytokines, depends on their maturation status. DC maturation can be induced by T cells (CD40 ligand) (108), pro-inflammatory molecules (IL-1 β , TNF- α , IFN- α , IFN- γ and PGE2) (109, 110) and DC-surface receptors involved in pathogen recognition (PRR) (110). The combination of different DC-maturation triggers determines which kind of T_H cell response is initiated (Figure 8).

Figure 8. DC vaccination strategy.

Monocytes derived from cancer patients (1) are differentiated into iDCs by different differentiation cocktails (2) followed by maturation, which is induced by cocktails containing proinflammatory cytokines, PGE2 or TLR-ligands (3). During maturation MHC class I and II membrane expression is upregulated and by different antigen loading techniques, DCs are induced to present antigens (4). These antigen-presenting DCs are reinjected into the patient (5), where they have to migrate into the draining lymph nodes (6). In these lymph nodes DCs interact with T cells and DC-cytokine secretion and expression of co-stimulatory molecules determines T_H cell polarization and CTL induction (7).

Activation of T cells by DCs depends on four independent signals inducing their differentiation and potentiating either their helper, killer or suppressive activity. These four signals include antigen presentation, which determines the specificity of the response; co-stimulation, determining the magnitude of the response; cytokine secretion, which is responsible for the type of immune response; and homing instructions that instructs effector T cells to migrate to the tumor site (111-113). For the induction of a specific T cell response, DCs should first be capable to efficiently present antigen. To this end, many *ex vivo* antigen-loading strategies have been developed, resulting in antigen presentation in MHC class I and/or II. These strategies include loading DCs with proteins (114), peptides, exosomes (115), viral vectors (116), plasmid DNA and RNA transfection (116-118), immune complexes (119) and tumor cell lysates (120, 121) (Figure 8). Fine-tuning of DC-maturation triggers, thereby influencing their cytokine profile, is of utmost importance for the efficacy of antitumor DC vaccination protocols. To induce an effective antitumor response T_H1 and T_H17 activation should be initiated whereas Treg induction should be avoided. Classical maturation cocktails often contain a combination of both $TNF-\alpha$ and $PGE2$. These DCs are most often used in clinical vaccination trials. However, over the past years increasing *in vitro* evidence suggests that TLR-ligand matured DCs might have a superior effect on T_H1 polarization and subsequent CTL induction which is based on their enhanced IL-12 producing capacity (110, 122, 123). Finally, DC-mediated T cell homing to the tumor site is a very poorly understood mechanism and manipulation of this mechanism is still under intensive investigation.

A large number of clinical phase I studies and smaller numbers of phase II and III studies with therapeutic DC vaccination protocols have been performed in human cancer patients. DCs have been successfully used to treat melanoma, lymphoma and renal cell carcinoma (124-128). They have proven to be safe and to induce tumor-specific T cells (129, 130). In 2010, the U.S. Food and Drug Administration (FDA) approved the first clinical vaccine preparation, which is used to treat prostate cancer (131). Additionally, in Switzerland the national public health organization has authorized a DC vaccination strategy for glioblastoma (132). Despite these encouraging developments, overall clinical response rates to DC vaccination strategies do not exceed 10-40% (133, 134). A few potential problems for the lack of improvement in survival have been proposed and are listed in Table I (4, 135).

In conclusion, DC vaccination strategies have been proven effective, however to improve response rates in clinical trials, new DC vaccination techniques should be analyzed and their effects must be studied in defined cancer patients with a low tumor load.

Table 1.

	Obstacles in DC vaccination therapy
1	Patients used in trials are heavily immunosuppressed by chemo- and radiation therapy and often have a large tumor burden (134).
2	Most injected DCs remain at the site of injection and do not migrate into the draining lymph nodes (136).
3	T cell stimulation by DCs needs to be directed towards induction of T _H 1 polarization and CTL activation instead of activation of Treg cells (137, 138).
4	Tumor suppressive effects may lead to T cell anergy, which is not easily overcome by DC vaccination (139).
5	Most DC vaccination strategies only target one single tumor antigen, whereas tumors are able to edit expression of immunogenic antigens.

Antigen therapy

T cells, B cells and plasma cells can be stimulated *in vivo* to produce antibodies or induce CTLs, by administration of antigens to cancer patients. These antigens are phagocytized and presented in MHC class I and II molecules by APCs (including DCs) to B and T cells (55) (Figure 9). Antigens can be administered as whole tumor cells, gene-modified tumor cells and long or short peptides (114). Important antigens used in clinical trials are MAGE, which is expressed by many tumors but mainly melanoma, WT-1 that is highly expressed in leukemia and various types of solid tumors, MUC1, which is expressed by almost all adenocarcinomas and NY-ESO-1, mainly expressed in melanoma, ovarian and lung cancer (140-142). Immune cell activating adjuvants are often additionally used to enhance the effect of antigen vaccination strategies (Figure 9).

Figure 9. Induction of active immune responses.

Induction of humoral and cellular immune responses can be induced by antigen administration. These and other immune responses can be enhanced by the simultaneous administration of TLR-ligands or cytokines. NK and T cells can additionally be activated by IL-2 and IL-12 stimulation.

Immune adjuvants, chemokines and cytokines

Adjuvants that stimulate molecular pathways in innate immune cells, enhancing tumor-associated inflammation and recruitment of immune cells, have been utilized in clinical trials (143, 144). The most common cells targeted in active immunotherapy are APCs (40). Among these cells, DCs are the most potent antigen presenting cells that can be targeted by adjuvant therapy. The function of DCs can be enhanced by different adjuvants, both organic and inorganic (145). In clinical vaccination strategies the most utilized inorganic adjuvant is alum (146, 147). Over the last decade however, organic adjuvants are also frequently used. The majority of these organic adjuvants contain Toll-like receptor (TLR) ligands that induce activation of DCs to engulf tumor-associated antigens and present

them to cells of the adaptive immune system (148, 149). This activation of DCs by TLR-ligands can be enhanced by cytokines.

TLRs are the best characterized pattern-recognition receptors that bind highly conserved pathogen-associated molecular patterns (PAMPs) (150), which are expressed either extracellularly (TLR1, TLR2, TLR4, TLR5 and TLR6) or intracellularly (TLR3, TLR7, TLR8 and TLR9) and are capable of binding different pathogens. The location of the TLRs (intra- or extracellular) is organized according to the infectious route by which different pathogens enter the cell.

The effect of TLR-ligands in cancer therapy was already demonstrated in the 19th century by William Coley, who showed that patients who developed bacterial infections after sarcoma surgery had visible regression of their tumors (151). Administration of TLR-ligands, both locally or systemically, has been shown to have potent anticancer effects (46, 152-155), which is evidenced by the effects of intravesicular injection of live bacilli Calmette-Guérin (BCG) in patients with bladder cancer (156). TLR-agonists can be used as monotherapy but also as adjuvants in immunotherapy and have been thought to overcome tolerance to tumor-self antigens (150, 152, 157). The antitumor responses are based on TLR-mediated DC activation, which leads to the production of chemokines and cytokines involved in recruitment of leukocytes and activation of NK cells and cytotoxic T cells (CTLs) (5, 7, 39). Additionally, TLR-ligands can directly induce apoptosis of tumor and stromal cells, by binding to these cells (158, 159).

DCs can not only be activated by the beforementioned TLR-ligands, also cytokines can be used to induce DC maturation, differentiation and activation. Attractive cytokines used for this purpose include Flt3L, GM-CSF and IFN- α . Administration of IFN- α in patients with chronic myeloid leukemia (CML) or renal cell cancer has been shown to induce effective antitumor immune responses mediated by both T and NK cells (5, 39).

Immune effector cells can also be targeted to induce antitumor responses. Important effector cells are NK, NKT and T cells (160). In addition to their cytolytic capacity these cells are efficient producers of IFN- γ , a cytokine that has tumoricidal effects and activates DCs, macrophages and T cells. NK, NKT and T cells can become activated both *in vitro* and *in vivo* by IL-2 and IL-12 stimulation (161, 162). Therefore these cytokines are used in many different anticancer therapies. IL-2 has been used to treat melanoma and renal cell

carcinoma patients, with an overall response rate of 15-20% (161). IL-12 is the main cytokine responsible for the induction of T_H1 responses and induces IFN- γ secretion by both T cells and NK cells (162). In turn, IFN- γ is a very potent DC-activating cytokine, thereby forming a positive feedback loop for enhancement of IL-12 production by DCs, which suggests that IL-12 might be a very potent adjuvant in cancer vaccines (162). Even though initial effects, characterized by substantial antitumor activity and complete remission in renal cell carcinoma (163, 164) and melanoma patients (165), were promising, administration of optimal doses of both cytokines proved to be toxic (18).

IFN- α is another cytokine involved in NK cell activation since it induces NK cell proliferation and augmentation of NK cell-mediated tumor cell lysis (166, 167). Additionally, it increases T cell-mediated killing of neoplastic cells (163) and enhances the ability of DCs to present antigen (43). IFN- α is used in many different malignancies including hematological malignancies and solid tumors (168, 169). Especially in CML, IFN- α therapy has been proven effective (170, 171), since it induces hematological remission in 60-80% of patients in early chronic phase CML. This clinical remission is correlated with upregulation of NK cell activity (164), presence of antibodies against CML-associated antigens (172) and presence of CML peptide-specific T cells (173).

Aim of the thesis

Immunotherapy has been reported to be successful only in a minority of cancer patients and therefore optimization is needed to increase clinical success rates. Dendritic cell vaccines for solid tumors as well as hematological malignancies need to be optimized at different levels. First, tumor-specific antigens need to be identified. Second, monocyte-derived DCs can be matured and differentiated by many different strategies which have different effects on the induction of immune effector cells. Additionally, interaction of DCs with other innate or adaptive immune cells is important to induce effector responses. This can either be achieved by DCs migrating into lymph nodes or by DC-mediated recruitment of immune cells. Activation of different subsets of immune effector cells by DCs is of importance to predict possible clinical outcome. Therefore, it is of significance to understand more about the biology of immune responses and to know which immune cells should be preferentially targeted to induce antitumor responses. Equally important is the effect of immunosuppressive factors, like PGE₂, produced by tumor cells and their microenvironment, on DC-mediated responses. This knowledge may lead to the administration of *ex vivo* developed effector cells, which can replenish the effector cell population, avoiding the negative effect of immunosuppressive factors. Finally, effective read-out systems of immune responses induced in patients need to be developed to assess therapy and predict possible clinical responses.

In this thesis, all of these topics are investigated and optimized to develop a combination of improved immunotherapeutic strategies to effectively fight cancer (Figure 10).

Figure 10. Aim and outline of this thesis.

To optimize DC vaccination protocols, tumor-specific antigens need to be identified. Monocytes can be isolated from blood, differentiated into immature DCs and matured by different stimuli to mature DCs. DCs are then loaded with tumor-associated antigens and injected into the patient. DCs, either have to migrate into the lymph nodes or can recruit immune effectors cells, thereby forming a tertiary lymphoid structure where they interact with effector cells. This interaction will either activate the patient's own effector cells or T cells that were developed or activated *ex vivo* and injected into the patient. The effector cells are then involved in tumor cell lysis. Techniques to evaluate induction of immune responses are crucial to follow up treatment efficacy. Numbers in red represent the chapters of this thesis in which the topic is addressed.

Outline of the thesis

In **chapter 2** we investigated the expression of underglycosylated MUC1 (MUC1-Tn/STn) in ovarian cancer, which could serve as tumor-specific antigen to be targeted in immunotherapy. Additionally, the mAb recognizing this epitope could possibly be used in diagnostics as well as antibody treatment. To optimize DC vaccination strategies, we evaluated the *in vitro* effect of different DC-maturation protocols on the induction of T cell responses in **chapter 3**. Not only effects on induction of adaptive immune responses of these differently matured DCs were assessed, but also their capacity to recruit and activate NK cells was evaluated in **chapter 4**. PGE2 is a potent immune suppressor, which is secreted by many solid as well as hematological malignancies. Paradoxically, PGE2 is often used in DC maturation protocols. In **chapter 5** we evaluated the effect of PGE2 on NK-DC interaction.

To evaluate the induction of humoral immune responses by DC-mediated immunotherapy, we developed a flow cytometry-based assay to detect human serum antibodies to MUC1 and underglycosylated MUC1, which is described in **chapter 6**. To enhance the effectiveness of DC vaccination and other forms of immunotherapy, *ex vivo* development of tumor-specific T cells that are not subjected to tolerance during development are of possible interest. Therefore we developed an *ex vivo* method to generate T cells, which is described in **chapter 7**. In **chapter 8**, a general discussion is presented, discussing the impact of our findings on improvement of cancer immunotherapy, with a special focus on the effect of NK-DC interaction in DC vaccination (Figure 10).

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Chapter

2

Expression of aberrantly glycosylated Mucin-1 in ovarian cancer

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Abstract

Aims: Mucin 1 (MUC1) is an important tumor-associated antigen (TAA), both over-expressed and aberrantly glycosylated in adenocarcinomas. The objective of this study was to examine the MUC1-glycosylation status in primary ovarian adenocarcinomas and metastatic lesions.

Methods and results: Paraffin-embedded tissue sections of 37 primary ovarian adenocarcinomas representing all histotypes (22 serous, 5 mucinous, 2 clear cell, 8 endometrioid), 4 serous borderline tumors with intraepithelial carcinoma, 7 sections of ovarian endometriosis and 13 metastatic lesions were analyzed by immunohistochemistry. Non-neoplastic ovarian surface epithelium and serous cystadenomas were used as controls. All epithelia expressed the MUC1 protein. Of primary tumors, 76% expressed the differentiation-dependent glycoform and 84% the cancer-associated glycoform (Tn/Sialyl-Tn-epitopes). In metastatic lesions this was 77% and 85%, respectively. Notably, in 57% of ovarian endometriosis and 75% of intraepithelial lesions, the cancer-associated MUC1 epitopes were expressed, whereas normal ovarian surface epithelium and serous cystadenomas did not express these epitopes.

Conclusions: The underglycosylated MUC1 epitopes are expressed by all histotypes of primary ovarian adenocarcinomas, by the vast majority of metastatic lesions and by possible ovarian cancer precursor lesions, whereas not by normal ovarian tissue. These results indicate that MUC1-associated Tn/STn-epitopes are important targets for immunotherapy and diagnostic imaging in ovarian cancer patients.

Introduction

Ovarian cancer is the second most common gynecological malignancy in developed countries, and the most common cause of death among women developing gynecological cancer (1, 2). The high mortality rate is ascribed to the fact that more than 60% of ovarian cancer patients already have advanced disease at diagnosis. Therefore, ovarian cancer is also referred to as “silent killer”. Metastasis as well as intra-abdominal growth are frequent, because the malignantly transformed ovarian surface epithelium is continuous with the peritoneal mesothelium (3).

The current treatment of ovarian cancer is surgical resection, followed by platinum-based chemotherapy. Despite advances in chemotherapy treatment, which have led to improved survival, relapse is still frequent in the majority of patients with advanced disease (4). To improve overall survival of ovarian cancer patients there is an urgent need for new treatment options. Immunotherapy has been demonstrated to be successful in the treatment of cancer (5). For antibody-mediated immunotherapy or cancer vaccines to be successful, identification of tumor-associated antigens (TAA), against which an immune response is induced, is of utmost importance. Up to this moment there have been few candidate-TAAs identified for ovarian cancer and clinical effects are limited (6).

An interesting TAA is Mucin 1 (MUC1), because it is overexpressed in most adenocarcinomas (7-9). MUC1 is a protein expressed on the apical surface of most simple, secretory epithelia and on a variety of hematopoietic cells (10, 11). MUC1 is a transmembrane mucin with an extracellular domain made up largely of 20 amino acid tandem repeats. After translation, the MUC1 protein becomes modified by extensive *O*-glycosylation. Within each tandem repeat, two serines and three threonines represent five potential *O*-glycosylation sites. The extent of glycosylation mainly depends on the expression of tissue-specific glycosyltransferases (8). Importantly, it has been demonstrated that in most adenocarcinomas and some hematological malignancies MUC1 is aberrantly glycosylated, has lost its apical expression and is secreted into the circulation (12-16). Due to aberrant glycosylation of MUC1 on tumor cells, new glycosylation-specific peptides are revealed, including the MUC1-associated Tn/STn-epitopes. These epitopes are generated when *O*-glycosylation is terminated immediately after the first N-acetylgalactosamine

(GalNAc) addition (Figure 1) (17, 18). The mechanisms responsible for Tn- and STn formation are still ill understood. It has been shown that alterations in ST6GalNAc-I transferase induces premature termination of glycosylation and subsequently enhances STn formation (19). Another documented mechanism involves somatic mutation of the *Cosmc* gene, a molecular chaperone regulating the enzyme responsible for the addition of galactose (Gal) to the first GalNAc (20). MUC1-associated Tn- and STn-epitopes, both recognized by mAb 5E5 (18, 21, 22), are potential TAAs, which can be targeted in immunotherapy but are also of importance in diagnostic imaging (17, 23).

Overexpression of MUC1 has been reported in ovarian cancer (24, 25). The expression of MUC1-associated Tn/STn-epitopes has been suggested (26), based on a similar expression pattern of MUC1 epitopes as well as Tn/STn-epitopes. However, the expression of MUC1-associated Tn- and STn-epitopes has never been proved.

In this study, we determined whether primary ovarian tumors and metastatic lesions express aberrantly glycosylated MUC1. Since the most widely accepted candidate for the origin of ovarian cancer is the ovarian surface epithelium (OSE) (27), we examined the MUC1 glycosylation status of OSE adjacent to the tumor in neoplastic ovaria and the epithelium of serous cystadenomas. Additionally, we analyzed MUC1 expression of ovarian endometriosis and serous borderline tumors with intraepithelial carcinoma/micropapillary growth, because both have been reported to be precursor lesions of invasive ovarian adenocarcinomas (28). We demonstrate that the MUC1-associated Tn/STn-epitopes are expressed on all different ovarian cancer histotypes and their metastases, while they are absent in normal ovarian tissue, rendering these epitopes potential targets for immunotherapy and immunodiagnostic imaging.

Materials and methods

Tissue samples

Paraffin-embedded tissue samples were obtained from files of the Department of Pathology, (Maastricht University Medical Center⁺) from 1990 to 2006. The study population consisted of 43 patients diagnosed with epithelial ovarian cancer. For 37 patients, primary tumors were analyzed. For 8 of these patients, matched metastases were analyzed. Of 5 other patients, only metastatic lesions could be analyzed. As a control, normal OSE adjacent to the tumor of 9 MUC1 positive tumors and 7 serous cystadenomas were analyzed.

The 37 primary ovarian tumors originated from different histotypes and comprised of 22 serous adenocarcinomas, 5 mucinous adenocarcinomas, 2 clear cell carcinomas, 8 endometrioid carcinomas. Additionally, 4 serous borderline tumors with intraepithelial carcinoma and 7 endometriosis lesions were analyzed. The 8 matched metastatic lesions were all typed as serous adenocarcinomas and the 5 non-matched metastatic lesions consisted of 4 serous adenocarcinomas and 1 mucinous adenocarcinoma.

Antibodies

MUC1 antibody 214D4 (purified from the supernatant of the 214D4 cell line (29)) was kindly provided by Dr. J. Hilkens (the Netherlands Cancer Institute, Amsterdam, the Netherlands). The mAb SM3 (supernatant (30)) was contributed by Prof. Dr. J. Taylor-Papadimitriou (Guy's Hospital, London, United Kingdom). MAb 5E5 (supernatant (21, 22)) was donated by Prof. Dr. H. Clausen (Department of Glycobiology, University of Copenhagen, Denmark). A detailed description of the specificities of the MUC1 antibodies used in this study has been published previously (18). Briefly, the mAb 214D4 recognizes human MUC1 irrespective of its glycosylation pattern, mAb SM3 recognizes the differentiation-dependent glycoforms and mAb 5E5 exclusively recognizes cancer-associated glycoforms of MUC1 (MUC1-associated Tn and STn) (Figure 1) (21). Although the mAbs SM3 and 5E5 both display cancer-associated reactivity (30), mAb SM3 also stains healthy human tissue as has been shown previously in the kidney and thymus (31, 32).

Figure 1. Glycosylation of tumor-associated MUC1.

Schematic representation of carbohydrate antigens, mAb and glycosyltransferases assayed in this paper. O-linked glycosylation is initiated by polypeptide N- acetylglucosaminyltransferases (a), adding N- acetylglucosamine (GalNAC) to serine (Ser) or threonine (Thr) of the polypeptide backbone, resulting in the Tn antigen, recognized by mAb 5E5. The Tn antigen may be sialylated by sialyltransferase ST6GalNAC-I (b), adding sialic acid (NeuAc) to the GalNAC moiety of Tn, resulting in the sialyl-Tn (STn) antigen, also recognized by mAb 5E5. If not sialylated, core 1 structures may be synthesized by galactosyltransferase Core 1 β 3-GalT (c), adding galactose (Gal) to the GalNAC moiety of Tn, resulting in the T antigen. Addition of further sugar moieties will result in the formation of Core 2 antigen formation, recognized by mAb SM3.

Immunohistochemistry

Paraffin-embedded tissue sections were deparaffinised by xylol (Merck, Darmstadt, Germany) treatment and rehydrated in decreasing concentrations of methanol (Merck). After rehydration, endogenous peroxidase was blocked (0.5% H_2O_2 in methanol) for 30 minutes and the slides were washed in PBS (Sigma-Aldrich, St. Louis, USA) supplemented with 0.05% Tween 20 (Merck). Specific staining of MUC1 was determined by incubation with primary anti-MUC1 mAbs or matched isotype controls overnight at 4°C. Afterwards, sections were rinsed with PBS/Tween 20 and incubated with Envision-HRP (DAKO, Glostrup, Denmark) for 20 minutes at room temperature. The color was developed by incubation for 3 minutes at room temperature using a substrate buffer containing 3,3'-diaminobenzidine (5 μ g/mL; DAKO) and 0.5% H_2O_2 (Sigma, Zwijndrecht, the Netherlands)

in PBS/Tween 20. Cytopins of the breast carcinoma cell line ZR75-1 (Department of Pathology, Maastricht University Medical Center[†]) proven to stain positive for all anti-MUC1 mAbs served as intra-experimental positive control for all antibodies (32). The histological images were analyzed by microscope (Carl Zeiss, Sliedrecht, the Netherlands) with an objective magnification of 10-20x. Digital pictures were taken by a Jenoptic camera (Carl Zeiss). The ovarian tumor slides were analyzed independently by five investigators (C.V.E.; F.B.; M.H.; W.G.; K.V.d.V.). Staining of cytoplasm and cell membranes was evaluated and cells were considered positive when at least one of these components showed positive staining.

Results

Expression of MUC1-associated Tn/STn-epitopes in ovarian cancer and adjacent healthy tissue

To determine the MUC1 glycosylation status, normal and malignantly transformed ovarian epithelium were immunoreacted with different MUC1 glycosylation-specific antibodies. Figure 2 shows a tissue section, containing normal OSE and malignantly transformed epithelium. Clearly, the MUC1 protein was expressed on the apical surface of normal ovarian epithelium, whereas the differentiation-dependent and cancer-associated glycoforms were not detected (Figure 2). In all 9 neoplastic ovarian tissue samples containing OSE, MUC1 expression of the normal epithelium was limited to the apical surface.

Strikingly, the malignantly transformed epithelium of the same ovary showed abundant expression of the MUC1 protein (Figure 2B), as well as the differentiation-dependent and cancer-associated glycoforms of MUC1, detected by mAb SM3 and 5E5, respectively (Figure 2C, D). Furthermore, in some malignant cells apical expression of MUC1 was lost, and MUC1 was detected on the entire cell surface (Figure 2D, arrows).

As an additional control of benign ovarian epithelium, serous cystadenomas were analyzed for expression of MUC1. All of these cysts (7/7) expressed the MUC1 protein

(mAb 214D4) and also 29% (2/7) expressed the differentiation-dependent glycoform, (mAb SM3) but none of them (0/7) the cancer-associated MUC1 glycoform (mAb 5E5).

Figure 2. MUC1 expression by healthy and malignant ovarian epithelium.

Expression of different MUC1 glycoforms on human ovarian tissue sections containing normal and malignant ovarian epithelium (serous adenocarcinoma). Sections were stained with mAb specific for MUC1 glycoforms. (A) Isotype control. (B) The glycosylated MUC1 isoform (mAb 214D4) was strongly expressed on the apical side of the normal epithelium, and on malignant transformed epithelium. (C) No expression of the differentiation-dependent glycoform of MUC1 (mAb SM3) in normal tissue while malignant transformed tissue stained positive. (D) The cancer-associated isoform of MUC1 (mAb 5E5) was not expressed on normal tissue, but was expressed on the malignant tissue. Loss of apical surface expression is indicated (arrows). * Normal ovarian epithelium, ** malignantly transformed epithelium.

Expression of MUC1 and cancer-associated MUC1 on different ovarian cancer histotypes

Since there are four different epithelial ovarian cancer histotypes, all were examined using the panel of MUC1 glycosylation-specific antibodies. The prevalence differs for each histotype, with the serous adenocarcinoma being the most common type of epithelial ovarian cancer, and the clear cell and endometrioid adenocarcinoma being rare (3). This was reflected in the number of tumors that was available for examination for each different histotype (22 serous adenocarcinomas, 5 mucinous adenocarcinomas, 2 clear cell carcinomas and 8 endometrioid adenocarcinomas). All ovarian cancer histotypes expressed MUC1 (37/37) (Table 1). The differentiation-dependent glycoform (mAb SM3) was found on 82% (18/22) of the serous, 60% (3/5) of the mucinous, 63% (5/8) of the endometrioid and on all of the clear cell (2/2) adenocarcinomas. The cancer-associated glycoform (mAb 5E5) was expressed on 86% (19/22) of the serous (Figure 3A), 80% (4/5) of the mucinous (Figure 3B), all of the clear cell (2/2) (Figure 3C) and on 88% (7/8) of the endometrioid adenocarcinomas (Figure 3D).

Table 1. Expression of the different MUC1 glycoforms on the different ovarian carcinoma histotypes.

Histotypes / mAb	214D4	SM3	5E5
Serous adenocarcinoma	100% (22/22)	82% (18/22)	86% (19/22)
Mucinous adenocarcinoma	100% (5/5)	60% (3/5)	80% (4/5)
Clear cell carcinoma	100% (2/2)	100% (2/2)	100% (2/2)
Endometrioid adenocarcinoma	100% (8/8)	63% (5/8)	88% (7/8)

In contrast to serous cystadenomas, ovarian endometriosis can serve as a precursor lesion of ovarian cancer, especially of the endometrioid and clear cell subtypes (27). Since most of the endometrioid adenocarcinomas express the MUC1 cancer-associated glycoform, we investigated the MUC1 glycosylation pattern of ovarian endometriosis. All endometriosis lesions expressed MUC1 (7/7) (Figure 4A), the differentiation-dependent glycoform (mAb SM3) was expressed by 57 % (4/7) and the cancer-associated glycoform (mAb 5E5) by 71% (5/7) of the endometriosis lesions (Figure 4B, C).

Additionally, serous borderline tumors with intraepithelial ovarian carcinoma, another precursor lesion of invasive ovarian adenocarcinoma, was examined for its MUC1 expression. All of these lesions expressed the MUC1 protein (4/4) (Figure 5A), 50% (2/4) the differentiation-dependent glycoform (Figure 5B) and 75% (3/4) the cancer-associated glycoform (Figure 5C).

In summary, the MUC1-associated Tn/STn-epitopes could be detected in tissue sections of all different ovarian cancer histotypes. Importantly, these epitopes were expressed on 86% of the serous adenocarcinomas, the most frequently diagnosed ovarian malignancy. For the other histotypes the number of tumor sections analyzed was too low to draw firm conclusions, but tumors as well as ovarian cancer precursor lesions expressed the cancer-associated MUC1 epitopes.

Figure 3. Expression of the cancer-associated glycoform of MUC1 on the different ovarian cancer histotypes. (A) Serous adenocarcinoma. (B) Mucinous adenocarcinoma. (C) Clear cell carcinoma. (D) Endometrioid adenocarcinoma. Detected with mAb 5E5.

Figure 4. Expression of different MUC1 glycoforms in endometriosis of the ovary. (A) The glycosylated MUC1 isoform (mAb 214D4), (B) differentiation-dependent glycoform of MUC1 (mAb SM3) (C) and cancer-associated glycoform of MUC1 (mAb 5E5).

Figure 5. Expression of different MUC1 glycoforms in a serous borderline tumor with intraepithelial carcinoma. (A) The glycosylated MUC1 isoform (mAb 214D4), (B) differentiation-dependent glycoform of MUC1 (mAb SM3) (C) and cancer-associated glycoform of MUC1 (mAb 5E5).

Expression of MUC1 and cancer-associated MUC1 in ovarian cancer metastases

As already indicated, ovarian tumors are prone to metastasize, therefore metastatic lesions were examined for their MUC1 expression. In 8 cases, it was possible to analyze both the primary tumor as well as the metastases. In the metastatic lesions, expression of MUC1 was detected in all (13/13) of the samples (Figure 6B). The differentiation-dependent MUC1 glycoform was detected in 77% (10/13) and the cancer-associated MUC1 glycoform in 85% (11/13) of metastatic lesions (Figure 6C, D). When the MUC1 expression profile of the metastases was compared with the profile of the primary tumor, the expression of MUC1 (mAb 214D4) was similar (100%). Additionally, in 63% (5/8) there was a similar expression profile of the differentiation-dependent MUC1 glycoform (mAb SM3) and even better results were obtained for the expression of MUC1-associated Tn/STn-epitopes (mAb 5E5), which showed comparable expression in all cases.

Figure 6. Expression of different MUC1 glycoforms in a metastatic ovarian cancer lesion in the omentum.

(A) Staining with isotype control. (B) The glycosylated MUC1 isoform (mAb 214D4), (C) differentiation-dependent glycoform of MUC1 (mAb SM3) (D) and cancer-associated glycoform of MUC1 (mAb 5E5) were expressed in the metastatic lesion.

Discussion

We demonstrate that aberrantly glycosylated, cancer-associated MUC1 is expressed *in situ* in most ovarian cancer tumors, but not in OSE and serous cystadenomas. Moreover, we show that the cancer-associated MUC1 epitopes are not only present in primary tumors, but also in ovarian cancer precursor lesions and in metastatic lesions. The expression of cancer-associated MUC1 epitopes in breast cancer has already been identified (21). Until now, the presence of cancer-associated MUC1 specific Tn- and STn-epitopes in ovarian cancer has been suggested, but not yet proven (26). Here we show that the cancer-associated MUC1 Tn/STn-epitopes, recognized by mAb 5E5, are indeed expressed in ovarian cancer and not in healthy ovarian tissue. In accordance with previous reports, the mAb SM3 also reacts with ovarian cancer tissue (30). Even though the mAbs SM3 and 5E5 both display this cancer-associated reactivity in ovarian cancer, mAb SM3 also reacts with healthy tissue as has been shown previously in thymus, salivary glands, sebaceous glands and the distal tubulus of the kidney (31, 32). This indicates that antibodies recognizing MUC1-associated Tn/STn-epitopes might have more potential to be used in ovarian cancer diagnostics and especially in immunotherapy.

Several studies have reported on the monoclonality of most epithelial cells in endometriotic cysts (33, 34) and therefore endometriosis is classified as a tumor-like lesion (28). The fact that the epithelium of ovarian endometriosis also expresses the MUC1-associated Tn/STn-epitopes, further supports the theory that endometriosis is actually a precursor lesion of ovarian cancer (27). Molecular events involved in the transformation of endometriosis have been studied extensively (27, 35) and decreased glycosylation of MUC1 may be a marker of this process. This is supported by the expression of MUC1-associated Tn/STn-epitopes by serous borderline tumors with intraepithelial carcinoma, which is confirmed to be a precursor of invasive serous adenocarcinomas. If antibodies recognizing the MUC1-associated Tn/STn-epitopes are suitable for discrimination between high and low risk endometriosis lesions, this will improve risk assessment and possibly therapy of younger women who want to preserve fertility. However, before being used in routine diagnostics, larger series of endometriosis

lesions and ovarian tumors have to be analyzed and related to clinical outcome in a comparative study.

MUC1 is immunogenic, both humoral and cellular immune responses against normal and underglycosylated MUC1 have been identified (15). Humoral immune responses have been confirmed by the detection of anti-MUC1 antibodies in the serum of healthy women and in women with ovarian cancer (16, 36). Anti-MUC1 antibody levels tend to be lower in cancer patients, which is possibly caused by the formation of immune-complexes with serum-MUC1 (37). These anti-MUC1 antibodies have been shown to mediate antibody-dependent cellular cytotoxicity (ADCC) of MUC1 positive cancer cells and to be protective in cancer development (38, 39).

Despite its immunogenicity (23, 29, 40), MUC1 also contributes to escape from tumor immuno-surveillance and metastasis formation. In tumor cells, MUC1 has an anti-adhesive function, which is not only of importance in cancer development (40), but might be responsible for a diminished adherence of immune effector cells to the malignant cells (41).

Immunotherapy will be essential to enhance immune responses to MUC1. MUC1 peptide vaccination or peptide-pulsed DC vaccination have shown to increase the amount of anti-MUC1 antibodies and induce proliferation of MUC1-specific cytotoxic T cells (15, 42-44). However, induction of a clinical effective cellular or humoral immune response to MUC1 has not yet been achieved. One of the reasons might be central tolerance to the mature MUC1 protein (21). Thymic medullary epithelial cells express the MUC1 protein, thereby most likely inducing negative selection of MUC1-specific T cells. However, the underglycosylated MUC1 isoforms are not present in the thymus, and therefore it was suggested by our group, that tolerance is induced only to the mature MUC1 and not the MUC1-associated Tn/STn-epitopes (32).

In this study, the underglycosylated MUC1-associated Tn/STn-epitopes were identified as potential TAAs, which could be used as targets for immunotherapy and immunodiagnostics in ovarian cancer patients and patients who are diagnosed with ovarian cancer precursor lesions. The expression of these epitopes by ovarian cancer and precursor lesions indicates that there are alterations in MUC1 processing and elucidation of the mechanism behind these alterations might increase our knowledge on ovarian

tumor biology. Cancer-specific MUC1 is expressed in 86% of all different ovarian cancer histotypes and in 85% of metastatic lesions, but not in normal ovarian tissue. Moreover, in the most frequent histotype, the serous adenocarcinoma, the percentage of tumors expressing the cancer-associated MUC1 is high (86%). Additionally, antibodies recognizing MUC1-associated Tn/STn-epitopes represent an effective diagnostic tool for the detection of ovarian cancer cells in tissue and fluids. Finally, since the MUC1-associated Tn/STn antibodies exclusively detect tumor cells, these antibodies could be effectively used for antibody-mediated immunotherapy and radio-labeling could be very useful for *in vivo* imaging and therapy.

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Chapter

3

Increased tumor-specific CD8⁺ T cell induction by dendritic cells matured with a clinical grade TLR-agonist in combination with IFN- γ

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Abstract

The limited response rate of cancer patients treated with dendritic cell (DC)-based vaccines indicates that vast improvements remain necessary. In many murine tumor models it has been demonstrated that the use of innate triggers (e.g. TLR triggers) in the maturation of DCs results in higher efficacy. However, as few of these innate triggers are generated clinical grade, there remains a great necessity to fill the gap between fundamental mouse studies and a clinical trial in humans. In the present study we used a TLR2/4-agonist (FMKp which is available clinical grade) in combination with IFN- γ (FI-cocktail) in the maturation of elutriated monocyte-derived DCs and compared it with the most used DCs in current clinical trials (TNF- α /PGE2, i.e. TP-cocktail). In addition to the assessment of CD4⁺ T cell polarizing capacity, we compared the quantity and intrinsic quality of induced CD8⁺ T cells of two different DC maturation protocols with cells from the same donor. Besides differences in the cytokine profile, which could be coupled to increased T_H1 and T_H17 polarization, we demonstrate in this study that FMKp/IFN- γ matured DCs are twice as effective in inducing cytotoxic T cells against known tumor antigens. Both DCs induced phenotypically equivalent effector memory CD8⁺ T cells that did not show a significant difference in their intrinsic capacity to kill tumor cells. These findings point to the therapeutic applicability of FI-DCs as superior inducers of functional antigen-specific T cells. Their increased chemokine secretion is suggestive of a mechanism by which these DCs may compensate for the limited migration observed for all *ex vivo* cultured DCs when applied in patients.

Introduction

Dendritic cells (DC) are very efficient initiators of potent immune responses *in vivo*. These cells are therefore attractive tools for cellular immunotherapy in the treatment of cancer and viral infections that evade regular immune surveillance (1-3). As a consequence, a range of methods to generate large amounts of DCs *ex vivo* has become available. They can be generated from a variety of sources, but DC differentiation from monocytes with a growth factor cocktail containing IL-4 and GM-CSF is the current gold standard (4, 5).

Translating these culture methods into good manufacturing practice guidelines has allowed the introduction of DC-based immunotherapy into clinical practice. Early DC vaccination trials have proven to be feasible, non-toxic (6) and the principle studies proved that DCs loaded with tumor antigens can induce immune responses (7, 8). However, the limited rate of tumor regression in clinical trials has indicated that mounting clinically effective immune responses using *ex vivo*-generated DCs is difficult (9). Among the causative factors for this limited response is definitely our limited understanding of the molecular signals driving effective T cell responses without simultaneous induction of suppressive T cells. Since DCs can easily be manipulated *in vitro*, most effort has been invested in finding a relation between DC maturation and T cell stimulation (10). The multitude of maturation cocktails used for superior vaccination design illustrates that the optimal stimulatory signal remains to be established. The most often used DC maturation cocktail in current applications includes TNF- α and PGE2 with or without IL-1 β and IL-6 (11). Although this cocktail generated fully mature DCs that are able to induce antigen-specific T cells and show migratory behavior *in vitro*, it has become clear that this cocktail is far from optimal (12). One of the most important disadvantages is the limited ability to mount potent T_H1 responses illustrated by the lack of IL-12 production, which presumably results from the effect of PGE2 that is added to stimulate DC migration (13-16). Many maturation cocktails have been proposed as alternative. One attractive option involves the use of microbial ligands and Toll-like receptor (TLR) agonists in particular (17). The TLR2/4 agonist FMKp in combination with IFN- γ has been shown to exert powerful DC-activation signals leading to high IL-12 secretion, T_H1 skewing and cytotoxic T cell induction (18, 19).

Numerous studies have pointed toward the necessity of PRR (Pattern of Recognition Receptors) triggering (TLR stimuli in particular) in DC maturation to obtain potent T cell responses, and this was correlated with potent antitumor responses in animal models (20-23). However, in the field of DC vaccination there is a big necessity to fill the gap between fundamental studies and a clinically approved trial. One tool which is available to fill this gap is performing *in vitro* experiments with primary human cells. These human studies are of particular relevance for the study of TLR-ligands as discrepancies in TLR expression and function between species have been reported (24). To date, only few TLR-triggering agents have been generated according to good manufacturing practice (GMP) allowing their use in the clinical practice. In addition, it also remains to be established how the induction of antigen-specific T cells is influenced by these ligands.

Few studies comparing differential DC maturation focus on CD8⁺ T cell heterogeneity (25, 26). In analogy with CD4⁺ T cell subsets, also functionally different CD8⁺ T cell subsets exist which exert opposite effects on T cell polarization *in vivo* (27, 28). These CD8⁺ subsets were designated T_C1 secreting IFN- γ and T_C2 secreting IL-4 (29, 30). In addition, CD8⁺ T cells can differentiate upon antigen recognition into either central (T_{CM}) or effector memory (T_{EM}) T cells. The factors that drive CD8⁺ T cells into these different phenotypes remain to be determined but the quantity and quality of stimulation by DCs and cytokines have been suggested as contributors (31). At present it remains unclear whether differential DC maturation can be exploited to generate different subsets of CD8⁺ T cells. To this end, we made a direct comparison of two clinically relevant DC maturation cocktails from the same DC preparations. The first cocktail consisted of a clinical grade preparation of membrane fractions of *Klebsiella pneumoniae* (FMKp) in combination with IFN- γ (FI-cocktail), whereas the second cocktail was composed of TNF- α and PGE2 (TNF- α /PGE2, i.e. TP-cocktail) which is most often used in current DC vaccination trials. These DCs were compared according to their phenotype, migratory behavior and cytokine secretion which was correlated to their T helper cell polarization and their differential ability to induce cytotoxic T cells. The data provide insight into DC-regulated control of T_H1/T_H17 versus T_H2 polarization, CTL phenotype and functionality, and establish FI-DCs as powerful inducers of antigen specific T cells.

Materials and methods

Dendritic cells

Immature DCs (immDC) were generated by ImmunoDesignedMolecules (Paris, France) from 5 HLA-A2⁺ leucopheresis products as previously described (17, 32) and frozen at 20 x 10⁶ cells per vial in 1.5 ml Iscove's Modified Dulbecco's Medium (Invitrogen Ltd., Paisley, UK) + 50% human serum (HS, Sanquin blood bank, Maastricht, The Netherlands) + 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich Co., St. Louis, MO, USA). As partners in a joint EU project, we obtained immDCs from IDM. Upon thawing, cells were left to recover in serum free AIM-V[®] containing differentiation cytokines. The following two days immDCs were matured using one of two maturation cocktails. The TP-maturation cocktail consisted of AIM-V[®] with IL-4 (500 U/ml; Strathmann Biotech AG, Hannover, Germany), GM-CSF (400 U/ml; Berlex, Richmond, CA, USA), TNF- α (1000 U/ml; Biosource, Camarillo, CA, USA) and PGE2 (18 μ g/ml Sigma) (33). The FI-cocktail contained IL-13 (50ng/ml; Biosource, Camarillo, CA, USA), GM-CSF (500 U/ml), IFN- γ (500 U/ml; Strathmann Biotech AG) and a TLR2/4 agonist (1 μ g/ml) (17). As TLR agonist, a GMP-grade lysate of *Klebsiella pneumoniae* was used (Pierre Fabre institute, Boulogne, France). This preparation contains membrane fractions (including the TLR2 ligand outer membrane protein A OmpA (34)) and proteoglycans (including the TLR4 ligand LPS) from *Klebsiella pneumoniae*.

Scanning electron microscopy

Glass coverslips (Miles Scientific, Naperville, IL, USA) were cut, transferred to a 24-well Plate and coated for 1 h with 50 μ g/ml fibronectin (Roche Diagnostics GmbH, Mannheim, Germany). After washing the coverslips, ImmDCs were allowed to adhere and mature in cocktail-containing medium for 3 h, followed by two PBS washes. Coverslips were fixed in 2.5% glutaraldehyde/PO₄ for 10 minutes, washed twice with PBS and analyzed with a XL30TMP microscope (FEI Company, Eindhoven, the Netherlands).

Flow cytometry

All antibodies for flow cytometry were obtained from BD Pharmingen (BD Biosciences, Erembodegem, Belgium) except anti-CCR7 (R&D Systems, Minneapolis, MN, USA) and

anti-Foxp3 (eBioscience, San Diego, CA, USA). MUC113-21 and MART-126-35 specific HLA-A*0201⁺ dextramers were purchased from Dako Cytomation (Glostrup, Denmark). Antibodies were used as either Fluorescein isothiocyanate (FITC), Pycoerytherin (PE), PE-Cy7, Peridinin chlorophyll protein (PER-CP) or allophycocyanin (APC). All antibodies were incubated at the proper dilution for 30 minutes at RT. Cells were fixed in 1% paraformaldehyde/PBS (PFA, Merck, Darmstadt, Germany). 7AAD was used to exclude dead cells from analyses that were all performed on a FACSCalibur (BD Biosciences).

Cytokine array

After 48 h maturation, cell-free supernatants of differentially matured DCs (10⁶ cells/ml) were harvested. Undiluted supernatants were analyzed on human cytokine antibody arrays VI-VIII (RayBiotech, Norcross, GA, USA) according to the manufacturer's instructions. After development, blots were photographed with a LAS-3000 imaging system (Fujifilm, Sint-Niklaas, Belgium). The spots were quantitated using AIDA Array Analysis Software (ImaGenes, Berlin, Germany). Quantitated chemiluminescence values of individual spots were normalized to the mean of 6 positive control spots after background (mean of 4 negative control spots) subtraction using the RayBio Human Cytokine Antibody Array C Series 2000 Analysis Tool (RayBiotech).

Cytokine secretion

Quantification of IL-5, IL-6, IL-10, IL-12, IL-17, TNF- α , IFN- γ TGF- β 1 and CCL19 in culture supernatants was performed using the respective ELISA kits obtained from Pierce Endogen (Rockford, IL, USA), R&D Systems, and Sanquin (Amsterdam, The Netherlands), and conducted according to the manufacturer's instructions. Absorbance was measured at 450 nm using a microtiterplate reader (BioRad, Hercules, CA, USA). Intracellular IFN- γ and IL-4 production of antigen-specific T cells was determined after coculture with peptide pulsed T2 cells for 4 h at 37°C in the presence of 1 μ g/ml Brefeldin A (BD Biosciences). After staining the extracellular antigens CD3 and CD8, cells were fixed and permeabilized using the Fix&Perm kit from Caltag Laboratories (Burlingame, CA, USA) followed by addition of anti-human IFN- γ and IL-4 (BD Biosciences) antibodies and incubation for 15 minutes at RT. Cells were fixed in 1% PFA/PBS and analyzed by flow cytometry.

Migration assay

Migration of mature DCs towards chemoattractants was measured using Transwell® plates (Corning Costar, New York, NY, USA). The lower cabinet was filled with 600 µl AIM-V® containing 250 ng/ml CCL19 or CCL21 (R&D Systems, Minneapolis, MN, USA). Triplicate inserts were filled with 100 µl 10^6 DC/ml, and migration proceeded for 4 h at 37°C / 5% CO₂. The migrated cells were collected from the lower compartment and counted using the Z1™ Coulter Counter (Beckman Coulter, Fullerton, CA, USA). To calculate the percentage of migrated cells, the total number of migrated DCs was divided by the total number of DCs in the upper well after subtraction of the mean number of spontaneously migrated DCs.

Priming culture

T cells were isolated from 9×10^8 PBMCs of the same leukapheresis product by binding them to fixed sheep red blood cells (SRBC). After density centrifugation, SRBC were removed from the T cell fraction by lysis in NH₄Cl and KHCO₃. DCs were pulsed in AIM-V® for 2 h with 40 µg/ml MUC113-21 peptide (LLLTVLTVV; NeoMPS, Strassbourg, France) or 40 µg/ml MART-126-35 peptide (ELAGIGILTV; NeoMPS) together with 5 µg/ml β2-microglobulin and 50 µg/ml PADRE (AGVAAWTLKAAA). Subsequently, 24×10^6 T cells were stimulated with 2×10^6 peptide pulsed DCs in AIM-V® containing 10 ng/ml IL-7 (Biosource, Camarillo, CA, USA). After 7 days, T cells were harvested, counted and restimulated with 2×10^6 pulsed DCs (20 µg/ml MUC1₁₃₋₂₁ or 20 µg/ml MART-1₂₆₋₃₅ with 1 µg/ml β2-microglobulin) in 24 ml AIM-V® supplemented with 20 U/ml IL-2 (Proleukin, Chiron Benelux BV, Amsterdam, The Netherlands). Medium with cytokines was refreshed every other day. On day 12 of the priming culture, T cells were collected and stained with CD8-PE and APC-labeled MUC1₁₃₋₂₁ or MART-1₂₆₋₃₅ dextramers (Dako Cytomation) for 1 h at RT. Cell suspensions were filtered through MACS pre-separation filters (Miltenyi, Utrecht, the Netherlands) and CD8⁺/dextramer⁺ cells were sorted with the FACSaria (BD Biosciences). Sorted cells were cultured for 7 days in presence of phytohaemagglutinin (0.2 µg/ml) and IL-2 (40 U/ml) on feeder mix consisting of irradiated (80 Gy) PBMCs and JY cells. Viability was determined by trypan blue exclusion.

51Cr-Release assay

The ability of sorted T cells to kill target cells was assayed using a standard chromium release assay. In short, antigen-specific T cells were incubated for 4 h at 37°C / 5% CO₂ with 10⁴ target cells, at an E:T ratio of 20:1. As target cells we used T2 (Tap1/2-deficient) and MUC1 positive tumor cell lines (MCF7, breast cancer; OVCAR-3, ovarian cancer and RPMI8226, multiple myeloma) were labeled with 75 µCi 51Cr-sodiumchromate/10⁶ cells (Amersham Life Sciences, Buckinghamshire, England). Target cells alone were used as negative control (spontaneous release) and target cells in the presence of 10% SDS (Bio-Rad Laboratories, Hercules, USA) as positive control (maximal release). Supernatants were collected and analyzed with a gamma counter. Specific kill was calculated as:

$$(\text{CPM}_{\text{sample}} - \text{CPM}_{\text{blanko}}) / (\text{CPM}_{\text{SDS}} - \text{CPM}_{\text{blanko}}) \times 100\%$$
Statistical analysis

To compare the percentages of antigen-specific T cells by both DCs (T+TP-DC and T+FI-DC, respectively), we normalized values to the percentages observed with TP-DCs (100%). The normalized percentage of T+FI-DCs was compared with this 100% value using the non-parametric Wilcoxon signed rank test. Cytokine secretion between cell populations was compared with paired T-tests. *P* values are given.

Results**Dendritic cell phenotype and cytokine production after 48-hour maturation**

For all experiments aliquots of immDCs were thawed and matured with either the TP-cocktail or the FI-cocktail (see M&M). Figure 1A shows representative scanning electron micrographs of both types of mature DCs (mDC) (*n*>20). TP-DCs exhibit a satellite morphology with long, unbranched dendrites whereas FI-DCs display a more elongated appearance with fewer but more branched processes.

Figure 1. Phenotypic characterization of TP- and FI-cocktail matured DCs.

(A) Scanning electron micrographs of differentially matured DCs after 48 h of maturation on fibronectin coated glass coverslips. Bars represent 10 µm. (B) Flow cytometric comparison of surface expression of HLA-molecules and maturation markers. Filled areas represent surface expression of TP-DC whereas bold lines show FI-DC. Representative data of 5 donors from 3 experiments are shown. All markers were upregulated compared to immDCs (data not shown). (C) Human cytokine protein arrays were performed using undiluted culture supernatant of TP- and FI-DC 48 h after maturation in a density of 10^6 cells/ml. Quantitated chemiluminescence values of individual protein spots were normalized to the intensity of control spots after background subtraction. This normalized protein expression is plotted for differentially matured DC in an X/Y graph. The solid diagonal line represents proteins found in equal amounts in both samples. Proteins are not annotated when they are less than 1.5 fold upregulated in either sample, meaning they are located between both dotted lines ($Y=1.5X$ and $Y=0.66X$) nor when their absolute expression is below 10x background (within grey box). (D) Quantitative comparison of production of CD4⁺ polarizing cytokines by TP- and FI-DC (48 h of maturation in a density of 10^6 cells/ml) as measured by ELISA. For IL-12 and IL-10, 3 leukapheresis products were tested twice in independent experiments. For TNF- α , values are given for both 0-48 h production and 6-48 h production (after wash). Paired T- test significance: IL-12 ($p<0.0001$), IL-10 ($p=0.03$), IL-6 ($p=0.002$) and TNF- α ($p=0.04$) (when DCs were washed at 6 h) and TGF- β ($p=0.18$).

To evaluate the phenotype of both differently matured DCs, we assessed by means of flow cytometry the expression of MHC class I and class II molecules (HLA-A2 and HLA-DR, respectively), costimulatory molecules (CD80, CD83, CD86 and CD40), CD1a, which is involved in presentation of non-peptide antigens, and DC characteristic integrin CD11c. Both mature DCs populations showed an upregulation of HLA-DR, CD80, CD83, CD86, and CD11c as compared to immDCs (data not shown). When comparing the expression levels of maturation markers between TP-DCs and FI-DCs (Figure 1B), we reproducibly noted a higher expression level of CD80 by FI-DC. Furthermore, we reproducibly (n=5) observed a slight upregulation of CD83 upon maturation, which was more pronounced in TP-DCs (Figure 1B). No differences were seen in the expression levels of HLA-A2, HLA-DR, CD86, CD40, CD11c and CD1a between the two DC populations, which was also reproducible.

Because DC-derived cytokines are considered key regulators in directing immune responses, we decided to screen whether both DCs differ in their production of different cytokines, therefore, a semi-quantitative antibody array analyzing 174 cytokines simultaneously was performed (Figure 1C). Quantitated chemiluminescence values of individual protein spots were normalized to the intensity of control spots (100% value) after background (0% value) subtraction. This normalized protein expression is plotted for differently matured DCs in an X/Y graph. The threshold for upregulation was set on 1.5. Eighty-six cytokines have spot intensities below the threshold (grey box in Figure 1C) in both samples and 33 factors are found in comparable amounts. Forty-seven out of 174 soluble factors have higher (>1.5 fold) spot intensities in the supernatant of FI-DCs, whereas only 8 are found at higher levels in the supernatant of TP-DCs. The identity of the differentially secreted protein factors and their fold increase in relative spot intensity are listed in Table 1. For 7 cytokines and chemokines we confirmed upregulation with ELISA (4 of which are shown in Figure 1D (IL-6, IL-12, IL-10 and TNF- α) and CCL5, CXCL10 and IL-4 (data not shown). In all cases the difference could be confirmed in more donors. Furthermore, the fold difference in spot intensity appeared to be an underestimation of the true fold difference in protein secretion when measured with ELISA in all cases (n=7). Among the factors found to be upregulated, several cytokines used in the maturation were identified. Indeed, IL-13, IL-4 and GM-CSF are among the brightest spots detectable since they are added in high concentrations to the supernatant. Importantly, TNF- α is not

exclusively found in the supernatant of TP-DCs (present in the maturation cocktail), but is actively produced by FI-DCs (Figure 1D). To assess whether TP-DCs are also able to produce TNF- α the maturation cocktail was washed away after 6 h and the potential production between 6 and 48 h was analyzed. In contrast to the supernatant of washed FI-DCs, no TNF- α was found in the supernatant of washed TP-DCs. Besides TNF- α , FI-DCs produce several cytokines involved in DC maturation (IL-1 β , TNF- α and IL-6), suggesting an autocrine maturation process. However, the most striking difference was a group of 15 chemokines (bold in Table 1) that potentially attract other immune cells and a number of cytokines involved in T cell polarization (IL- 6, IL-10, and IL-12) and T cell support (IL-7 and IL-15) that were found in higher quantities in the supernatant of FI-DCs as compared to TP-DCs.

Table 1. Comparison of differentially produced cytokines by TP-DC and FI-DC.

Proteins overexpressed in TP-DC		Proteins overexpressed in FI-DC			
<i>IL-4</i>	68.6	IL-1 β	78.1	CCL15	4.7
TIMP-2	2.7	IL-13	76.5	TNF- β	4.6
TIMP-4	2.1	IL-15	60.4	Leptin	3.8
CXCL16	2.0	CCL20	41.5	NT-3	3.4
Angiogenin	1.9	IL-10	32.5	IL-6	3.4
CD14	1.8	IL-1 α	30.5	SCF	3.2
SCF R	1.6	M-CSF	27.3	IL-1ra	2.9
MMP9	1.5	CCL13	24.8	CXCL9	2.9
		CCL5	22.8	PDGF-BB	2.6
		Oncostatin M	18.8	IGFBP-1	2.5
		CCL8	18.5	IL-12p40	2.4
		CXCL11	17.2	IGFBP-4	2.2
		IL-7	17.0	IGF-1 SR	2.0
		CCL1	16.2	PDGF-AA	1.9
		CCL2	14.5	TNF- α	1.8
		CCL3	11.7	IL-16	1.8
		IL-12p70	8.6	CXCL10	1.7
		CXCL5	8.3	NAP-2	1.7
		CCL7	7.9	TGF- β 1	1.6
		IFN- γ	7.8	IL-2RA	1.6
		Activin A	7.0	TGF- β 3	1.6
		LIGHT	6.7	CCL11	1.6
		IL-2	6.3	ALCAM	1.5
		CXCL1	4.8		

Cytokines indicated in italics are those added to the maturation cocktail and their high levels of expression validate the assay. Factors indicated in bold are chemokines potentially involved in immune effector cell attraction. Numbers represent the fold increase in relative spot intensity.

To study T cell polarizing cytokines quantitatively, these cytokines were measured in DC supernatants by ELISA. As shown in Figure 1D, FI-DCs show higher IL-12, IL-10, IL-6 and TNF- α (when DCs were washed at 6 h) production as compared to TP-DCs. Both DCs secrete low levels of the Treg inducing cytokine, TGF- β .

Effect of the maturation cocktail on the migratory program of DCs

For DCs to migrate, they have to be equipped with the appropriate set of chemokine receptors, whose expression is reprogrammed during the maturation process. To investigate the influence of both maturation cocktails on the chemokine receptor expression, we analyzed CCR6 and CCR7 expression. Both TP- and FI-DCs express low but reproducible levels of CCR7 (Figure 2A), but no CCR6 (data not shown) after 48 h of maturation.

Figure 2. Effect of the maturation cocktail on the migratory program of DCs.

(A) Flow cytometric evaluation of CCR7 surface expression of TP- and FI-DCs. Dotted histograms represent isotype staining, whereas the filled histograms represent CCR7 staining of the DCs. (B) At different time points after the induction of DC maturation (0, 16, 44 and 68 h) DCs were harvested to assess their migratory capacity. 10^5 DCs were transferred to the upper compartment of a transwell and allowed to migrate to a CCL19/21 gradient for 4 h. After 4 h, migration was stopped and cells were counted. For every donor the percentage of DCs that migrated is plotted ($n=3$ or 4 for all time points except 68 h ($n=1$)).

Because CCR7 is the key chemokine receptor that regulates trafficking of DCs to the lymph node, we compared their migratory behavior towards the CCR7 ligands; CCL19 and CCL21 in transwell assays. To exclude the possibility that both maturation cocktails induce migration with different kinetics, we followed their migration at different time points after

maturation. ImmDCs were CCR7 negative, and did not migrate towards the chemokine gradient (data not shown). TP-DCs showed an increased migration through the transwell upon maturation while FI-DC did not migrate at any time-point (Figure 2B).

T_H cell induction by DCs cultured with different maturation cocktails

The combination of cytokines present during the priming of naive T cells by DCs influences their polarization. Since TP- and FI-DCs display different cytokine profiles including polarizing cytokines, both DCs were pulsed with class I-restricted peptides and a pan HLA-DR binding peptide (PADRE) and were used to prime autologous T cells. To analyze whether both DCs differ in their CD4⁺ T cell polarizing capacity the cytokine profile of T cell cultures stimulated with both DCs after 7 days was compared. IL-5 secretion was only observed in coculture experiments with TP-DCs (in 4/5) and not in cocultures with FI-DCs. This suggests that at least some T_H2 polarization was induced by the former DC (Figure 3A), which is supported by our observation of a small but reproducible percentage of CRTH2⁺ CD4⁺ cells only identified when stimulated with TP-DCs (Figure 3B). CRTH2 is the prostaglandin D2 receptor and is considered the best surface marker for the selective staining of T_H2 cells (35). T cells stimulated with FI-DCs, on the other hand, produce more IFN- γ (in 4 out of 5 experiments), suggesting stronger T_H1 polarization induced by these DCs. This is supported by the observation of a higher percentage of CD4⁺ T cells with a high expression of CXCR3, which is characteristic for T_H1 polarized cells but is not as specific as CRTH2 for T_H2 cells (data not shown).

To determine if both DCs induce T_H17 cells, we examined whether IL-17 was produced. In the supernatant of T cells stimulated with FI-DCs, higher quantities of IL-17 were found (Figure 3A), indicating that these DCs are more capable of inducing T_H17 cells. Suppressive cytokines such as TGF- β and IL-10 were found in comparable amounts in both T cell cultures (Figure 3A).

As DCs can induce Tregs *in vitro*, we compared a possible Treg induction by both DC populations by FACS analysis. CD4⁺ Tregs are characterized by a high expression of CD25 and they express the transcription factor Foxp3, which is required for their regulatory function (36). On day 0, very low numbers of Tregs (CD25 and Foxp3 double positive cells) (<0.05%) were observed. Both DCs are capable of inducing Tregs, as approximately 1% of

CD4⁺ T cells were also Foxp3⁺ 7 days after the first stimulation with peptide-pulsed DCs (Figure 3C). No consistent difference in Treg induction could be found between TP- and FI-DCs.

Figure 3. Differential CD4⁺ T cell polarizing capacity of TP- and FI-DCs. 24x10⁶ T cells were stimulated for 7 days with 2x10⁶ TAA-peptide- and PADRE-pulsed DCs in presence of IL-7. (A) Cytokine profile of the induced T cells after DC-T cell coculture as measured in the supernatant taken after 7 days of coculture (n>4). At this time point presence of DCs could be no longer detected. Paired T-test significance: IFN- γ (p=0.12), IL-5 (p=0.08), IL-17 (p=0.06), IL-10 (p=0.76) and TGF- β (p=0.82). (B) Surface expression of CRTH2 as surface marker of T_H2 polarized cells on CD4⁺ T cells (gated on CD3⁺) 7 days after stimulation of naive T cells with TAA-peptide- and PADRE-pulsed DCs. (C) Induction of Foxp3⁺CD25⁺CD4⁺ and CD3⁺ T cells at day 7 after stimulation. Gated on CD3⁺CD4⁺ cells. These data are representative for 3 individual experiments with different donors.

FI-DCs induce more antigen-specific T cells as compared to TP-DCs

To compare the induction of tumor antigen-specific T cells, both mDCs were pulsed with HLA-A2-restricted immunodominant epitopes of the tumor-associated antigens (TAA) Mucin-1 (MUC1₁₃₋₂₁) or Mart-1 / Melan-A (MART-1₂₆₋₃₅) and the pan HLA-DR binding peptide (PADRE). Figure 4A shows the average cell numbers during T cell stimulation assays and no increase in total T cell number was observed during the initial priming with

either mDC. Upon restimulation, a strong proliferative response was observed in both conditions, but no significant difference in the absolute T cell numbers was measured.

Figure 4. Induction of antigen-specific CD8⁺ T cells by TP- and FI-DCs.

24×10^6 T cells were stimulated with 2×10^6 TAA peptide- and PADRE-pulsed DCs in presence of IL-7. After 7 days, cells were counted and restimulated with autologous DCs in the presence of IL-2. On alternate days medium was refreshed and fresh IL-2 was added. (A) Average cell counts ($\pm$ SD) at day 0, 7, 9 and 12 during T cell stimulation assay ($n=7$). (B) Quantitation of the percentage of MUC₁₃₋₂₁- and/or MART₁₂₆₋₃₅-reactive T cells at day 12 measured by dextramer staining on CD8⁺ cells (representative for $n=7$). (C) Comparison of the percentage of MUC₁₃₋₂₁- and MART₁₂₆₋₃₅-specific T cells generated with TP- and FI-DCs at day 7 (left panel) and day 12 (right panel). The upper 2 lines in both figures represent 2 stimulation assays with MART₁₂₆₋₃₅-pulsed DCs, whereas the other 5 were performed with MUC₁₃₋₂₁-pulsed DCs. Paired samples are connected with a dotted line (*P<0.05).

To assess whether both mDCs differed in their capacity to induce antigen-specific T cells, we quantified the number of antigen-specific CD8⁺ cells by dextramer staining on various days. In all individuals tested, we observed a higher starting frequency of MART-1₂₆₋₃₅-specific T cells compared to MUC1₁₃₋₂₁-specific T cells. Possibly, for this reason, higher numbers of MART-1₂₆₋₃₅-specific T cells were obtained throughout the stimulation assays. Figure 4B shows dextramer staining of T cells after two stimulations. Although there was no difference in the overall proliferative response with either DC, there was a difference in the percentage, and the absolute numbers, of antigen-specific T cells generated. After the first stimulation (day 7), significantly more antigen-specific T cells were observed when stimulation was performed with FI-DCs than with TP-DCs (Figure 4C). In 6 out of 7 experiments, more than double the absolute number of antigen-specific T cells after one stimulation was seen. After the second stimulation there was still a significant difference, and a two-fold increase was found in 5 out of 7 experiments (Figure 4C).

Both DCs induce effector memory antigen-specific T cells with IFN- γ but no IL-4 production

As quantity and density of stimulation and the cytokine microenvironment may influence the fate of memory T cell differentiation, we analyzed whether the different DCs influenced CD8⁺ T cell specialization. CD8⁺, antigen-specific T cells generated with both DCs stained positive for CD45RO and CXCR3 (Figure 5A), and negative for CD45RA (data not shown) and CCR7 (Figure 5A). This indicates that the generated antigen-specific T cells were of the T_{EM} and not the T_{CM} phenotype.

In addition to the T_{EM} and T_{CM} phenotype, antigen-experienced CD8⁺ T cells can be classified as T_{C1}, T_{C2} and T_{C0} based on their cytokine secretion. To determine the subtype, CD8 and dextramer double positive T cells were sorted from DC-T cell coculture experiments, incubated with peptide loaded T2 target cells and analyzed for cytokine expression. Upon antigen recognition, MUC1₁₃₋₂₁-specific T cells accumulated intracellular IFN- γ and no IL-4 accumulating T cells were found in any condition (Figure 5B). When MUC1₁₃₋₂₁ reactive T cells were stimulated with T2 cells loaded with an irrelevant (MART-1₂₆₋₃₅) or no peptide, only a very small percentage of IFN- γ accumulating cells was identified. This intracellular staining was confirmed by IL-4 and IFN- γ ELISA (3 donors; data

not shown). Similar data were observed when evaluating MART-1₂₆₋₃₅ reactive T cells (data not shown). These data indicate that, despite the difference in cytokine secretion and in the induction of antigen-specific T cells, both DCs induce antigen-specific T_H1 cells rather than T_H2 cells.

Figure 5. Phenotypic characterization of antigen-specific CD8⁺ T cells.

(A) CD45RO, CCR7 and CXCR3 staining of T cells on day 11. CD45RO and CCR7 are plotted against dextrans for antigen-specific T cells and were gated on CD8⁺ cells. CXCR3 was double stained with CD8, and gated on the living, low FSC/SSC cells. Left panels represent T cells stimulated with TP-DCs and right panels represent T cells stimulated with FI-DCs. (B) Antigen-specific T cells were sorted at day 12 from T cell stimulation assays. After 7 days of recovery on feeder cells in presence of IL-2, cells were stained or intracellular IL-4 (left panel) and IFN- γ (right panel) after stimulation (5 h) with peptide-pulsed (grey histograms) or non-pulsed (open histograms) T2 cells. Cells were gated on CD3⁺ and CD8⁺. Isotype controls overlap with non-pulsed T2 cells (not shown). (C) Fas and FasL expression of antigen-specific T cells on day 12 of the T cell stimulation assay. Cells were gated on CD3⁺ and CD8⁺. Stainings are representative of at least 3 independent observations.

One of the cytolytic mechanisms by which antigen-specific T cells can induce cell death in target cells involves the induction of FasL on the surface of cytotoxic T cells (37). This bystander mechanism may account for high background killing in *in vitro* cytotoxicity assays. Therefore, FasL-expression was routinely checked on antigen-specific T cells. Prior to cell sorting and cytotoxicity assays, all specific T cells stained negative for FasL (Figure 5C). In some experiments when a third stimulation was performed, expression of FasL was observed on the majority of antigen-specific T cells (data not shown). This was associated with reduced viability of T cell cultures and high background killing of target cells (data not shown). In contrast to the regulation of FasL, all T cells retained a constitutive expression of Fas throughout the stimulation assay (Figure 5C).

The intrinsic capacity of MUC113-21 reactive T cells to kill target cells is not influenced by differential DC maturation

To test whether differentially matured DCs influence the functionality of antigen-specific T cells, we compared their capacity to kill target cells. To exclude possible regulation by the CD4⁺ and the CD8⁺ compartment, antigen-specific T cells were sorted based on 7AAD⁻/FSC and gated on CD8⁺/MUC1₁₃₋₂₁⁺ cells. The second sorting gate and post-sort purity is shown in Figure 6A. The minimal post sort purity was >90%. After a recovery and expansion period, T cells were evaluated for their capacity to kill various target cell lines. As shown in Figure 6B, MUC1₁₃₋₂₁ pulsed T2 cells are killed by MUC1₁₃₋₂₁⁻ specific T cells while unpulsed T2 cells or MART-1₂₆₋₃₅ pulsed are not (data not shown). No differences in the amount of killing by T cells generated with either DC were seen. In addition, we showed, by studying lysis of MUC1 expressing HLA-A2⁺ tumor cell lines, that these T cells are also able to recognize naturally presented MUC1. Consistent with previous reports, we did not see lysis of the ZR-75.1 cell line (38). All other cell lines displayed varying levels of lysis when incubated with MUC1₁₃₋₂₁ reactive T cells, indicating that the MUC1-specific T cells can recognize the MUC1-peptide presented by tumor cell lines in the context of HLA-A2 (Figure 6C). Although there was considerable interexperimental variability regarding the percentage of lysis of these cell lines, we did not observe (within one experiment) consistent differences between T cells generated with differentially matured DCs.

Figure 6. Sorting of antigen-specific T cells and comparison of their killing capacity of various target cells.

(A) Day 12 T cell cultures were collected and stained with dextramers and with antibodies recognizing CD3 and CD8. Left and middle panels show representative examples of the sorting gate and the post sort purity. The right panel shows an example of the purity of the T cell cultures after 7 days of recovery on feeder cells. The minimal purity of these cultures to perform killing assays was set at 80% but as generally > 90%. (B) Chromium release assays of MUC₁₃₋₂₁-pulsed and non-pulsed T2 cells with sorted MUC₁₃₋₂₁ reactive T cells generated with TP-DC (black squares) and FI-DC (open squares). Data are calculated as % of maximal release when cells are exposed to 10% SDS (n=3). (C) Chromium release assays of MUC1⁺ HLA-A2⁺ tumor cell lines by MUC₁₃₋₂₁ reactive T cells generated with TP-DC (black squares) and FI-DC (open squares). Due to the limited amount of sorted T cells, not all the cell lines could be included in one experiment. Consequently, results of 3 independent experiments are combined.

Discussion

The successful clinical application of DCs as anticancer therapies requires detailed understanding of the molecular events associated with DC maturation and their manipulation to optimize immune responses. Although many murine studies have shown that the concept of DC vaccination works, it cannot be directly translated into clinical practice (1). Therefore, it is of the utmost importance to translate the experimental work into clinically approved protocols in humans. To date human studies have shown only limited success (10). To investigate how the use of toll-like receptor agonists influences the quality and quantity of antigen-specific CD8⁺ T cells in humans, clinical grade monocyte-derived DCs were matured with IFN- γ in combination with a membrane fraction of *Klebsiella pneumoniae* containing TLR2/4 agonists (FMKp, which is available clinical grade) (39). Phenotypic characterization combined with intracellular cytokine staining showed no difference in the specific subset of cytotoxic T cells that was induced with these different DCs. All antigen-specific CD8⁺ T cells are characterized as CCR7⁻, CD45RO⁺ and CXCR3⁺ T_{EM} cells. Both DCs induce predominantly antigen-specific CD8⁺ T cells with IFN- γ but no IL-4 secretion, which is consistent with a T_C1 phenotype. This characterization is further supported by the observation of activation-induced cell death in these cultures after a third stimulation, which was often associated with the induction of FasL on these T cells. Human T_C1 clones have been shown to display a higher susceptibility to activation induced cell death as compared to T_C2 clones (29). In addition, we observed no obvious difference in the killing capacity of sorted TAA-specific CD8⁺ T cells on the basis of equal cell numbers. This is supported by the observation that differentially matured DCs induce TAA-specific T cells with similar avidity (40). Taken together, these data indicate that the profound difference in cytokine profile of TP- and FI-DCs does not influence the intrinsic quality of generated T cells as both DCs induce phenotypically and functionally equivalent antigen-specific T cells. However, FI-DCs usually induced twice as many TAA-specific CD8⁺ T cells as compared to TP-DCs. Since this suggests that FI-DCs will also induce a larger CTL response in patients, it is relevant to elucidate the causative mechanism.

The first mechanism why more antigen-specific T cells were observed with FI-DCs may be due to the cytokine profile. It has been demonstrated that IL-12 not only affects the

differentiation of T_C1 cells (30) but also stimulates their expansion. In addition to IL-12, also other factors implicated in homeostatic proliferation such as IL-7 and IL-15 (41) are produced in higher quantities by FI-DCs. This indicates that in contrast to TP-DCs, FI-DCs are equipped with a cytokine profile which favors the rapid proliferation of CD8⁺ memory T cells.

A second possible contributing factor to the observed difference in T cell induction may be differential T_H polarization by both DCs. In the T cell stimulation assay CD4⁺ T cell activation was increased by the addition of a panHLA-DR binding peptide (PADRE), which is also proposed as adjuvant for vaccination trials (42). We observed that PADRE facilitates mounting CD8⁺ T cell responses. However, by comparing surface markers differentially expressed by T_H1 and T_H2 cells [CRTH2 and CXCR3; reviewed in (43, 44)] and the cytokine profile of T cell cultures we show that PADRE does not replace the need of T cell polarization. Our observations of IL-5 secretion and CRTH2 positive CD4⁺ cells only in stimulation assays with TP-DCs suggest that with these DCs at least some T_H2 polarization occurs. In contrast, the higher percentage of CXCR3⁺ CD4⁺ T cells upon stimulation with FI-DCs together with a high IFN- γ production is indicative of a more potent T_H1 skewing and the increased amount of IL-17 in these cultures indicates that there is also T_H17 polarization. These differences in T cell polarization are consistent with the cytokine profile of both DCs. FI-DCs produce more IL-12, favoring T_H1 polarization, and the high production of IL-6 and IL-1 β in combination with small amounts of TGF- β 1 (in contrast to TP-DC) has been shown to favor T_H17 differentiation (45, 46). Increased T_H1 polarization has already been coupled with increased CD8⁺ T cell responses, but with regard to T_H17 induction it remains to be determined how they influence cytotoxic T cell induction. It also remains debatable whether the induction of T_H17 cells is beneficial in cancer. T_H17 cells can contribute to tumor growth by stimulating tumor-associated inflammation, but are also suggested to induce antitumor responses via an IFN- γ dependent mechanism (47, 48). DCs have been shown to expand functional Tregs *in vivo* and the nature of the DC maturation stimulus has been shown to determine the efficacy of Treg induction *in vitro* (13). Because PADRE has been proven to reverse chemotherapeutic-induced immune suppression in vaccinated mice, it is relevant to study whether PADRE prevents Treg induction in humans. However, with both DCs, a small but significant population of

CD4⁺CD25⁺Foxp3⁺ T cells was observed 7 days after the first stimulation as compared to the starting population. Although Foxp3 was recently shown also to be transiently expressed by newly activated T cells (49), it is unlikely that such cells are present 7 days after stimulation. At present, we are performing functional assays to study the consequences of Treg induction.

To mount an effective antitumor response DCs are required to interact with the appropriate lymphocytes in the draining lymph node. CCR7-dependent trafficking to this anatomic location is considered essential for successful DC vaccines. Despite the difference in migratory potential of both DCs in transwell studies, recent *in vivo* trafficking studies have shown that only 2-3% of subcutaneously injected TP-DCs traffic towards draining lymph nodes in melanoma patients (50), which is comparable to nodal trafficking of FI-DCs in multiple myeloma patients using a similar vaccination regimen (51). Therefore, it still remains to be determined whether differentially matured DCs from one patient differ in their migration to lymph nodes and whether *in vitro* assays predict this migratory behavior. Besides trafficking to lymph nodes, DCs have to interact with appropriate lymphocytes, which are also chemokine driven. We show that differential maturation of DCs results in large differences in chemokine secretion. FI-DCs produce at least 15 chemokines in higher quantities as compared to TP-DCs, including important T cell attracting chemokines such as CCL19, CCL5 and CXCL10, which may ultimately lead to preferential attraction of different immune effector cells.

Taken together, we demonstrated that maturing DCs with a combination of TLR2/4 agonists (which is available clinical grade) plus IFN- γ induces stronger T_H1/T_H17 skewing and a two-fold higher number of antigen-specific T_{EM} killer cells than TNF- α plus PGE2 matured DCs. In addition, we show that FI-DCs have a higher production of 15 chemokines, suggesting that these cells are more potent attractors of other immune cells when administered *in vivo*. Importantly, injection of peptide-loaded FI-DCs alone has not been very successful in metastatic melanoma patients (52). This does not preclude the use of FI-DCs in the clinic but illustrates that for clinical success, other requirements than only DC quality such as tumor type, antigen selection and loading strategy, overriding immunosuppression, angiogenesis inhibition, among others, need to be manipulated. The present study demonstrates that TLR2/4 ligand plus IFN- γ matured DCs are superior

inducers of functional antigen-specific CD8⁺ T cells in comparison to the most often used DC in clinical trials. It can therefore be speculated that the induction of twice the number of antigen-specific T cells by the same number of injected DCs *in vivo* will result in a stronger antitumor response. Partly based on these results, a clinical trial is planned in multiple myeloma patients in which TLR2/4 plus IFN- γ matured DCs will be directly compared with DCs matured with the more conventional TNF- α plus PGE2 cocktail.

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Chapter

4

***Klebsiella pneumoniae*-triggered DCs recruit human NK cells in a CCR5-dependent manner leading to increased CCL19-responsiveness and activation of NK cells**

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Abstract

Besides their role in destruction of altered self-cells, NK cells have been shown to potentiate T cell responses by interacting with DCs. To take advantage of NK-DC crosstalk in therapeutic DC-based vaccination for infectious diseases and cancer, it is essential to understand the biology of this crosstalk.

We aimed to elucidate the *in vitro* mechanisms responsible for NK cell recruitment and activation by DCs during infection. To mimic a bacterial infection, DCs were exposed to a membrane fraction of *Klebsiella pneumoniae* (FMKp), which triggers TLR2/4. DCs matured with these bacterial fragments can actively recruit NK cells in a CCR5-dependent manner. An additional mechanism of DC-induced NK cell recruitment is characterized by the induction of CCR7 expression on CD56^{dim}CD16⁺ NK cells after physical contact with FMKp matured DCs, resulting in an enhanced migratory responsiveness to the lymph node-associated chemokine CCL19. Bacterial fragment matured DCs do not only mediate NK cell migration but also meet the prerequisites needed for augmentation of NK cell cytotoxicity and IFN- γ production of which the latter contributes to T_H1 polarization.

Introduction

NK cells are important effector cells in the innate immune response against virally infected or malignantly transformed cells and their cytotoxicity is regulated by a delicate balance of inhibitory and activating signals (1). Recent studies suggest that the interplay between NK cells and DCs, the specialized antigen presenting cell of the innate immune system (2), is critical in shaping the adaptive immune response (3). This concept originates from several lines of evidence including: the discovery of NK cells colocalizing with DCs in the T cell areas of lymph nodes (4, 5), the coupling of NK cell recruitment to lymph nodes with the induction of more potent T_H1 skewing (3), and the identification of NK cell subpopulations with helper properties (6). Although the exact mechanisms of NK-DC interaction remain to be elucidated, increasing evidence supports the importance of bidirectional NK-DC crosstalk (7, 8).

On the one hand, NK-DC crosstalk is characterized by the capacity of activated NK cells to induce DC maturation with elevated IL-12p70 production and subsequently an increased capacity to induce T_H1 and CTL responses (9). This NK-induced DC maturation depends at least in part on soluble factors such as TNF- α (10) and IFN- γ (11) as well as on engagement of the natural cytotoxicity triggering receptor 30 (NKp30) (12). Moreover, NK cells control the quality of the adaptive immune response by NKp30-mediated lysis of immature or inadequately matured DCs (13), enabling only fully mature DCs to migrate into lymph nodes and subsequently prime T cells. On the other hand, DCs are able to induce NK cell proliferation, augmentation of cytotoxicity and cytokine secretion (8). The DC-induced modulation of cytokine production by NK cells requires a strong immunological synapse between the two cell types and both DC-derived soluble factors (e.g.: IL-12 and IL-18) as well as contact-dependent factors (e.g.: IL-15 and NKG2D ligands) are implicated in this crosstalk (6, 14, 15). Mouse models have revealed a DC-dependent mechanism of NK cell recruitment into the lymph nodes. One of the functions of this recruitment is to provide IFN- γ , necessary for efficient T_H1 skewing (3). In humans, the mechanism for DC-dependent NK cell recruitment into inflamed lymph nodes remains to be unraveled and it is unclear whether chemokine profiles of activated DCs during infection match the prerequisites necessary for NK cell activation.

Klebsiella pneumoniae (*K. pneumoniae*) is an extracellular bacterium that induces activation of the innate immune system. *In vivo* mouse studies have revealed that endogenous IL-12, produced by alveolar macrophages, is a critical component of the antibacterial host defense against *K. pneumoniae* as blocking of IL-12 is associated with increased mortality (16). CXCL10 mediated NK as well as T cell recruitment and IFN- γ production has been reported to be essential to control *K. pneumoniae* infection (16, 17). This is supported by a study on CpG triggering during *K. pneumoniae* infection. CPG treated mice showed increased infiltration and activation of NK cells providing an early source of IFN- γ , which was associated with decreased mortality (18). NK cells are not only activated by other immune cells triggered by *K. pneumoniae*, but they are also equipped themselves with pattern recognition receptors (PRR) suitable for direct binding of the *K. pneumoniae* membrane protein OmpA, which triggers NK cells to produce IFN- γ and the antibactericidal peptide α -defensin (19).

In this study, we addressed the question, whether DCs triggered with fragments of *K. pneumoniae* display the ability to recruit and activate NK cells, ultimately leading to T_H1 polarization. Additionally, we set out to elucidate which mechanisms are responsible for these processes. To mimic a bacterial infection, DCs were matured by a membrane fraction of *K. pneumoniae* (FMKp), which is a lysate of the outer membrane fraction of *K. pneumoniae* (*K. pneumoniae*) containing the DC-triggering agents LPS and outer membrane protein A (OmpA), which signal through TLR 4 and TLR2, respectively (20). For its proven additional effect on upregulation of co-stimulatory molecules and modulation of cytokine secretion, IFN- γ was added to the FMKp maturation cocktail (21). This study led to the identification of chemokines and cytokines responsible for NK-DC crosstalk and we describe two possible mechanisms of NK cell recruitment into the inflamed lymph node. These *in vitro* observations are of relevance in unraveling the *in vivo* biology of NK-DC crosstalk.

Materials and methods

Cell isolation

Mononuclear cells from peripheral blood of healthy donors were isolated by density gradient separation using lymphoprep (Axis-Shield). NK cells were negatively selected by immunomagnetic cell separation (Miltenyi Biotec GmbH). CD4⁺ T cells were isolated using RosetteSep (StemCell Technologies). Naive CD4⁺ T cells were negatively selected by depleting CD45RO⁺ cells with immunomagnetic cell separation (Miltenyi Biotec GmbH). The purity of isolated populations exceeded 95% as determined by flow cytometry.

Generation of DCs

DCs were prepared from peripheral blood-derived monocytes, as previously described (22) and as control, highly pure monocyte fractions were obtained by elutriation from leukapheresis products of healthy volunteers, approved by the local medical, ethical committee of Maastricht University Medical Center⁺. Differentiation of monocytes was induced by 6 days of culture in AIM-V[®] medium (Gibco Life Technologies) containing 2000 U/ml IL-4 (Strathmann Biotech) and 400 U/ml GM-CSF (Berlex) or 50 ng/ml IL-13 (Biosource) and 500 U/mL GM-CSF. DCs were matured for 6-24 h in AIM-V[®] containing either 1000 U/ml TNF- α (Biosource) and 18 μ g/ml PGE2 (Sigma-Aldrich) or 500 U/ml IFN- γ (Strathmann Biotech), and 1 μ g/ml FMKp (Pierre Fabre). For maturation with single TLR2 and TLR4 ligands, DCs were matured in the presence of the same amounts of IL-4, GM-CSF and IFN- γ as IFN- γ /FMKp matured DCs supplemented with 10 μ g/ml Zymosan (InvivoGen) and/or 10 μ g/ml LPS (InvivoGen).

Flow cytometry

All antibodies for flow cytometry, except anti-IL-15 and CCR7 (R&D Systems), were obtained from BD Biosciences. Antibodies were used in Fluorescein isothiocyanate, Pycoerytherin, Peridinin chlorophyll protein or allophycocyanin. Cells were incubated with antibodies at proper dilutions for 30 minutes at RT. Analyses were performed on a FACSCalibur (BD Biosciences) and analyzed with BD CellQuest[™] Pro Software (BD Biosciences).

Migration assay

Migration of NK cells was analyzed using 5,0 μm Transwell[®] plates (Corning Costar). The lower cabinet contained 600 μl cell free DC supernatant or AIM-V[®] containing 250 ng/ml CCL19 (R&D Systems). Triplicate inserts were filled with 100 μl containing 5×10^5 NK cells, migration preceded for 16 h at 37°C / 5% CO₂. Migrated cells were counted using the Z1™ Coulter Counter (Beckman Coulter). To block migration, NK cells were incubated for 1 h with blocking antibodies for CCR5 (BD Biosciences), CCR7 or CXCR3 (R&D Systems) preceding migration. The contact-dependent increase in NK cell migration to 250 ng/ml CCL19 (R&D Systems) was determined after separating the NK cells from the NK-DC coculture by positive selection of DCs on HLA-DR (Miltenyi Biotec).

Chemokine array

Undiluted cell-free supernatants (24 h of maturation of 10^6 cells/ml) were analyzed on the human chemokine antibody array I (RayBiotech) according to manufacturer's instructions. Chemokine blots were photographed with a LAS-3000 imaging system (Fujifilm) and spots were quantified using AIDA Array Analysis Software (ImaGenes). Quantitated chemiluminescence values of individual spots were calculated using the Chemokine Antibody Array C Series 2000 Analysis Tool (RayBiotech).

Cytokine and chemokine secretion

Quantification of IL-18, IL-12, CCL5, CXCL10 and CCL19 in 24 h DC maturation supernatants was performed using ELISA (MBL International Corporation and R&D Systems) according to manufacturer's instructions. Absorbance was measured at 450 nm using a microtiter plate reader (BioRad).

Cytokine production by NK cells

Intracellular IFN- γ was determined after 16 h NK-DC coculture in the presence of 1 $\mu\text{g}/\text{ml}$ Brefeldin A (BD Biosciences). After extracellular staining, cells were fixed, permeabilized using PermWash (BD Biosciences) and stained with anti-human IFN- γ (BD Biosciences). Cells were fixed in 1% PFA/PBS and analyzed by flow cytometry. Quantification of IFN- γ production was performed using IFN- γ ELISA kit (Sanquin) in 24 h cell free supernatant. NK

cell activation was blocked by preincubating cell-free DC supernatant for 1 h with 0,5 µg/ml anti-IL-12 (R&D Systems) and 4 µg/ml anti-IL-18 (MBL International Corporation). For NK-DC contact-dependency, NK cells (10⁵ cells/well) were separated from DCs (2 x 10⁵ cells/well) by culturing the cells in different compartments of a 0,4 µm Transwell® plate (Corning Costar).

T_H cell priming

DCs (4 x 10⁴ cells/well) were cocultured for 24 h in the presence or absence of NK cells (5 x 10⁴ cells/well), before they were coated with 1 ng/ml Staphylococcal Enterotoxin B (SEB, Sigma-Aldrich) for 1 h. SEB-coated DCs, were washed and placed in culture with CD45RA+/RO-CD4+ T cells (10⁵ cells/well). On day 4, 20 U/ml rhIL-2 (Proleukin, Chiron Benelux BV) was added to the cultures. At day 10 the expanded T_H cells were washed, plated in 96-well plates (10⁵ cells/well), and stimulated with PMA/ionomycin (BD Biosciences) for 4 h in the presence of 1 µg/ml Brefeldin A (BD Biosciences). Intracellular IFN-γ staining was analyzed by flow cytometry.

NK cell cytotoxicity assay

NK cell cytotoxicity was analyzed by a flow cytometry-based kill assay as described (23). Briefly, NK cells were incubated for 24 h with either DC supernatant, medium alone or medium containing 1000 U/ml IL-2 (Proleukin). Target cells (Raji) were labeled with 3'-diiododecyloxacarbocyanine (DiO) according to the manufacturer's instructions (Sigma). Target cells (10⁴ cells) were incubated with effectors at various effector:target (E:T) ratios for 12 h, each ratio in triplicate. Percentages of killed target cells (PI⁺ DiO⁺) were determined by flow cytometry. Percentages of specific lysis were calculated: (% PI positive target cells - % spontaneous PI positive cells)/ % of vital cells) x 100.

Statistics

The statistical significance of differences between experimental samples was determined using Student's t test for paired samples or ANOVA for multiple samples. Significance was accepted at the $P < 0.05$ (*) and $P < 0.01$ (**) levels. Data were analyzed using Prism software version 5.00 (GraphPad Software, Inc.).

Results

DC-dependent NK cell recruitment is CCR5-mediated

To investigate whether NK cells are involved in the immune response against bacteria, we tested the ability of DCs to induce NK cell migration. A bacterial infection was mimicked by triggering monocyte-derived DCs with FMKp. As a control, DCs were matured with the pro-inflammatory mediators TNF- α and PGE2 in the absence of bacterial triggers. In addition to FMKp, also purified, single TLR ligands were used to investigate whether these individual pathways dominate induction of NK-DC interaction. Therefore DCs were matured with either Zymosan (TLR2 ligand) or LPS (TLR4 ligand) in the presence of IFN- γ . Moreover, the combination of both single TLR-triggering agents was used to determine a possible synergism.

IL-4/GM-CSF differentiated DCs were matured for 6 h in the presence of either TNF- α /PGE2 or IFN- γ /FMKp after which the cells were extensively washed and transferred to serum-free medium without maturation stimuli. Washing was performed to avoid altered NK cell migration due to activation by the maturation cocktail. For the TNF- α /PGE2 and IFN- γ /FMKp matured DCs 6 h maturation irreversibly triggered maturation as evidenced by the upregulation of MHC class II and costimulatory molecules (Supporting Information Figure 1), which was comparable to expression of these markers detected after 48 h maturation (24). Filtered, cell-free, DC supernatant was harvested after 18 h of culture and transferred to the lower compartment of a Transwell®. Since NK cell migration is more reproducible at longer migration periods (Supporting Information Figure 2), recruitment assays were performed over a period of 16 h. NK cell migration to a CCL19 gradient was used as a positive control to normalize donor and interexperimental variability. After 16 h of NK cell migration, the supernatant of the IFN- γ /FMKp matured DCs contained significantly more NK cells as compared to the supernatant of the TNF- α /PGE2 matured DCs (Figure 1A). We did not observe a selective enrichment of CD56^{bright} nor CD56^{dim} NK cells in the lower well, suggesting that there was no preferential recruitment of either NK cell subset (data not shown). No significant difference in the number of migrated NK cells was detected when medium was present instead of the supernatant of TNF- α /PGE2 matured DCs (data not shown), indicating that these DCs fail to attract NK cells.

To identify the chemokines responsible for the observed NK cell recruitment by IFN- γ /FMKp matured DCs, chemokine arrays were performed on DC supernatants. The chemiluminescence values of the chemokine spots were quantified by densitometry, normalized to control spots and plotted in XY graphs (Figure 1B). DC maturation induced by IFN- γ /FMKp led to an enhanced (>1.5 fold increase in chemiluminescence) secretion of 11 chemokines as compared to TNF- α /PGE2-induced maturation. These chemokines included CCL5, CXCL10 and CCL19 to which NK cells can respond (25-27). Quantification with ELISA confirmed that these chemokines were indeed produced by IFN- γ /FMKp matured DCs and not by TNF- α /PGE2 matured DCs (Figure 1C). To evaluate whether the chemokine production was induced by TLR-triggering or IFN- γ stimulation, DCs were matured by either FMKp or IFN- γ alone. Maturation with IFN- γ does neither induce DCs to produce CCL19 nor CCL5, however IFN- γ stimulates DCs to secrete CXCL10 (Figure 1D). FMKp is sufficient to trigger DCs to produce all three cytokines, albeit in lower concentrations as compared to maturation in the presence of both FMKp and IFN- γ , evidencing their additive effect.

Additionally, other TLR ligands were used to induce DC maturation and induction of chemokine production was compared with the production by IFN- γ /FMKp matured DCs (Figure 1D). In the presence of IFN- γ , zymosan, LPS and the combination of both TLR-ligands, triggered DCs to produce CCL19 and CXCL10, although at significantly lower amounts as compared to FMKp/IFN- γ matured DCs. Zymosan in contrast to LPS was not able to induce CCL5 production by DCs. No additive effect on chemokine secretion was observed when TRL2 and 4 were stimulated simultaneously. To evaluate if the differences in chemokine production between different TLR-triggered DCs influenced NK cell recruitment, supernatant of zymosan/IFN- γ and LPS/IFN- γ triggered DCs, matured by the same maturation and washing procedure as the IFN- γ /FMKp matured DCs were used in NK cell migration assays. Migration data revealed that IFN- γ /FMKp matured DCs recruited more NK cells than DCs matured with either FMKp or IFN- γ alone (Figure 1E). Moreover, FMKp/IFN- γ matured DCs also recruited significantly more NK cells than zymosan/IFN- γ matured DCs (Figure 1E). Furthermore, LPS/IFN- γ matured DCs, do not differ from IFN- γ /FMKp matured DCs in their capacity to recruit NK cells even though they produce lower amounts of chemokines.

Figure 1. CCR5-dependent recruitment of NK cells by IFN- γ /FMKp matured DCs.

Freshly isolated human NK cells and undiluted, filtered supernatants of IL-4/GM-CSF differentiated and TNF- α /PGE2 or IFN- γ /FMKp matured DCs were used for analysis. (A) Percentage of NK cell migration towards supernatants of TNF- α /PGE2 and IFN- γ /FMKp matured DCs. Data were calculated as percentage of migrated NK cells, normalized to NK cell migration towards a CCL19 gradient (250 ng/ml). Data show mean + SEM of 7 different donors; bars. * $P < 0.05$, paired t-test. (B) Human chemokine protein arrays of DC supernatant. Quantitated chemiluminescence values of individual protein spots are represented. Normalized protein expression is plotted in an X/Y graph and only 1.5-fold upregulated proteins are annotated. Data are representative of results from 3 donors. (C) Quantitative comparison of CCL5, CCL19 and CXCL10 production by IFN- γ /FMKp and TNF- α /PGE2 matured DC as measured by ELISA in at least 5 different donors. * $P < 0.05$, ** $P < 0.01$, paired t-test.

Figure 1. CCR5-dependent recruitment of NK cells by IFN-γ/FMKp matured DCs (continued).

(D) Chemokine production of IFN-γ/FMKp, IFN-γ, FMKp, Zymosan/IFN-γ, LPS/IFN-γ and Zymosan/LPS/IFN-γ matured DCs, combined data of at least 5 different donors. Data show mean + SEM, *P<0.05, **P<0.01, ANOVA. (E) Percentage of NK cell migration induced by supernatants of IFN-γ/FMKp, IFN-γ, FMKp, Zymosan/IFN-γ, LPS/IFN-γ and Zymosan/LPS/IFN-γ matured DCs. Data show mean + SEM of 7 different donors. *P<0.05, **P<0.01, ANOVA. (F) Percentage of NK cell migration towards DC supernatant after blocking the chemokine receptors CCR5, CXCR3 or CCR7. Data are calculated as percentage of migrated NK cells normalized to NK cell migration towards medium (background). Data show mean + SEM of 4 different donors. **P<0.01, paired t-test.

To establish which chemokines are involved in NK cell recruitment by IFN-γ/FMKp matured DCs, corresponding chemokine receptors on NK cells were blocked (CCR5 for CCL5, CXCR3 for CXCL10 and CCR7 for CCL19). Only blocking of CCR5 reduced NK cell migration significantly (Figure 1F). No significant reduction was observed with a CCR7-blocking antibody, which is like anti-CCR5 an IgG2a antibody and therefore serves as an isotype control.

In conclusion, DCs triggered with bacterial fragments of *K. pneumoniae* produce many chemokines in substantial amounts, including CXCL10, CCL19 and CCL5, and are able to recruit NK cells in a CCR5-dependent manner.

DC-dependent induction of CCR7 expression on NK cells increases NK cell migratory responsiveness to lymph node-related chemokines

In steady state conditions the CD56^{bright}CD16⁻ NK cell subpopulation has been reported to express CCR7 (27), which allows them to migrate into lymph nodes (28). However, in response to IL-18-producing monocytes, induction of CCR7 has also been observed on CD56^{dim}CD16⁺ NK cells (6). To investigate whether IFN- γ /FMKp matured DCs also induce CCR7 expression on NK cells, both cell types were cocultured. After 24 h NK cells were harvested, and CCR7 expression was evaluated using flow cytometry. Besides CCR7 expression on CD56^{bright} NK cells, CCR7 was also observed on the CD56^{dim} NK cell subpopulation after coculture with IFN- γ /FMKp matured DCs (Figure 2A), which was significant in seven different donors (Figure 2B; $P < 0.01$). When NK cells were physically separated from DCs by a Transwell[®] (pore size 0,4 μm), CCR7 expression was still induced, albeit on a lower percentage of NK cells (Figure 2B). This indicated that the induction of CCR7 expression was predominantly contact-dependent.

To test if this induction of CCR7 expression resulted in an increased migration towards lymph node-related chemokines, NK cells were isolated from NK-DC cocultures and tested for their capacity to migrate towards CCL19. Compared to freshly isolated NK cells, NK cells from NK-DC cocultures showed a significantly increased migratory responsiveness ($P < 0.01$; average migration index of 1.25) towards CCL19 (Figure 2C).

These data indicate that, upon contact with DCs triggered with bacterial fragments, CCR7 expression is induced on a subpopulation of NK cells, increasing their migratory responsiveness to CCL19, which is considered to be a lymph node-homing chemokine.

Figure 2. DC-dependent CCR7 induction on NK cells, inducing increased migratory responsiveness to CCL19.

Freshly isolated human NK cells and undiluted, filtered supernatants of IL-4/GM-CSF differentiated and IFN- γ /FMKp matured DCs were used for functional and phenotypic analysis. (A) Percentage of CCR7⁺ NK cells after culture for 24 h with AIM-V[®] medium, supernatant of IFN- γ /FMKp matured DCs or IFN- γ /FMKp matured DCs as detected by flow cytometry and gated on CD56⁺CD3⁻ NK cells. Representative data out of 6 donors are shown. (B) Percentage of CCR7⁺ NK cells after 24 h coculture with IFN- γ /FMKp matured DCs. Data show mean + SEM of 7 different donors. **P<0.01, paired t-test. (C) Migration index of NK cells, cocultured with IFN- γ /FMKp matured DCs and separated by magnetic bead separation, migrating for 16 h toward CCL19. Data show mean + SEM of 4 different donors. **P<0.01, paired t-test.

NK cell activation by IFN- γ /FMKp stimulated DCs enhances NK cell cytotoxicity and induces IL-12/IL-18-mediated IFN- γ production, resulting in T_H1 polarization

Recent data have shown that, lymph node-homing NK cells assist in T_H1 polarization (3, 29). Since IFN- γ /FMKp matured DCs were able to recruit NK cells, we investigated whether these DCs were also able to induce IFN- γ production by NK cells. To quantify the percentage of NK cells producing IFN- γ , intracellular IFN- γ staining was performed on NK cells cultured in DC supernatant (Figure 3A). A small subpopulation (about 2%) of NK cells accumulated IFN- γ when cultured for 16 h in the supernatant of IFN- γ /FMKp matured DCs (Figure 3B). Among the IFN- γ -accumulating NK cells we identified NK cells with both, high

and intermediate expression of CD56. Though small in number, the IFN- γ accumulating NK cell subpopulation was capable of producing detectable amounts of IFN- γ as measured by ELISA (Figure 3C). Induction of IFN- γ secretion of NK cells by DCs depends on the additive effect of IFN- γ and FMKp during DC maturation, since neither of these stimuli alone could induce as much IFN- γ production by NK cells as IFN- γ /FMKp matured DCs (Figure 3D). After activation by IFN- γ /FMKp DCs, the complete NK cell population showed upregulation of the NK cell activation markers CD69 and CD25 (Supporting Information Figure 3). Notably, NK cells cultured in the supernatant of the TNF- α /PGE2 matured DCs neither accumulated IFN- γ nor showed upregulation of activation markers.

To evaluate whether DCs triggered by single TLR-ligands, also induce NK cell activation, NK cells were incubated for 16 h in the supernatant of DCs matured with zymosan, LPS or the combination of both in the presence of IFN- γ . NK cells incubated with supernatant of these TLR-triggered DCs showed significantly lower production of IFN- γ than NK cells incubated with supernatant of IFN- γ /FMKp matured DCs (Figure 3D).

Two crucial soluble cytokines responsible for NK cell activation are IL-12 and IL-18, which act interdependently in a two-signal mechanism (6). To examine if these cytokines are involved in the observed IFN- γ production, their presence in the supernatant of IFN- γ /FMKp and TNF- α /PGE2 matured DCs was determined. IFN- γ /FMKp matured DCs produced IL-12p70 as well as IL-18, albeit with a large donor variation (Figure 3E). In contrast, TNF- α /PGE2 matured DCs did neither produce IL-12p70 nor IL-18. Since activation of NK cells by DCs depends on the additive effect of IFN- γ and FMKp, IL-12 production after maturation with these triggers individually was examined. DCs produced IL-12 after FMKp triggering but not after IFN- γ triggering, however, the combination of FMKp and IFN- γ had a synergistic effect on IL-12p70 production (Figure 3F). When production of IL-12 by DCs matured with other TLR2 and TLR4 ligands was evaluated, it was found that LPS, in contrast to zymosan, induced IL-12 production and also the combination of LPS and zymosan did, however, the amount produced by these DCs was significantly lower than that of IFN- γ /FMKp matured DCs (Figure 3F).

Figure 3. IL-12p70 and IL-18 dependent activation of NK cells by IFN- γ /FMKp matured DCs.

Freshly isolated human NK cells and undiluted, filtered supernatants of IL-4/GM-CSF differentiated and TNF- α /PGE2 or IFN- γ /FMKp matured DCs were used for analysis. (A) Percentage of NK cells accumulating IFN- γ after activation for 16 h by supernatant of TNF- α /PGE2 and IFN- γ /FMKp matured DCs, as detected by flow cytometry and gated on CD56⁺CD3⁻ NK cells. Representative data of 5 independent experiments are shown (B) and combined data of 5 different donors data show mean + SEM. **P<0.01, ANOVA. (C) Quantitative comparison of IFN- γ production by NK cells as measured by ELISA. Data show mean values of 10 donors. **P<0.01, ANOVA. (D) Quantitative comparison of IFN- γ production by NK cells activated for 16 h by supernatant of IFN- γ , FMKp, Zymosan/IFN- γ , LPS/IFN- γ and Zymosan/LPS/IFN- γ matured DCs. Data show mean + SEM of at least 5 different donors. **P<0.01, ANOVA. (E) Quantitative comparison of production of IL-12p70 and IL-18 by TNF- α /PGE2 and IFN- γ /FMKp matured DCs as measured by ELISA. Data show mean values of at least 8 donors. *P<0.05, **P<0.01, paired t-test. (F) Quantitative comparison of production of IL-12p70 by IFN- γ , FMKp, Zymosan/IFN- γ , LPS/IFN- γ and Zymosan/LPS/IFN- γ matured DC as measured by ELISA. Data show mean + SEM of 9 different donors. *P<0.05, ANOVA.

Figure 3. IL-12p70 and IL-18 dependent activation of NK cells by IFN- γ /FMKp matured DCs (continued).

(G) Surface IL-15 presentation *in trans* by DCs matured by TNF- α /PGE2 or IFN- γ /FMKp as detected by flow cytometry. Representative data out of 3 different donors are shown. (H) Quantitative comparison of IFN- γ production by NK cells activated for 16 h by supernatant of IFN- γ /FMKp matured DCs, after neutralizing IL-12p70 and/or IL-18, as measured by ELISA. Representative data of 3 independent experiments are shown. *, $P < 0.05$; **, $P < 0.01$.

Upon physical contact with IFN- γ /FMKp matured DCs, NK cells produce slightly elevated amounts of IFN- γ as compared to NK cells cultured in DC supernatant (Supporting Information Figure 4), indicating that predominantly soluble factors, with minor contribution of contact-dependent factors, mediate IFN- γ secretion by NK cells. This phenomenon was observed in both autologous as well as Killer cell Immunoglobulin-like Receptor- (KIR) mismatched NK-DC co-cultures. A candidate cytokine responsible for this contact-dependent increase of IFN- γ production is IL-15, which can be presented *in trans* by DCs (15, 30). Therefore expression of IL-15 by IFN- γ /FMKp and TNF- α /PGE2 matured DCs was evaluated by flow cytometry. Notably, IFN- γ /FMKp DCs express more surface bound IL-15 (Figure 3G).

The dependency of NK cell activation on DC-derived IL-12/IL-18 was examined by neutralizing both cytokines with the respective neutralizing antibodies. This resulted in a decreased IFN- γ production after neutralizing IL-18 and a complete block of IFN- γ secretion after neutralizing IL-12p70 (Figure 3H).

To investigate whether the observed DC-induced NK cell activation by IFN- γ /FMKp matured DCs contributes to T_H1 polarization, T cell stimulation assays were performed in the presence or absence of NK cells. Therefore, IFN- γ /FMKp matured DCs were coated with *Staphylococcus Enterotoxin B* (SEB) and cocultured for 10 days with naive CD4⁺ T cells

in either the presence or absence of NK cells. When NK cells were present, a significantly higher percentage of CD4⁺ T cells produced IFN- γ (Figure 4A; $P < 0.01$), which was reproducible in four donors (Figure 4B).

Figure 4. DC-dependent NK cell activation induces T_H1 responses.

IFN- γ /FMKp matured DCs differentiated by IL-4/GM-CSF were cultured for 24 h in the presence or absence of freshly isolated NK cells, before they were pulsed with Staphylococcal Enterotoxin B (SEB) and cocultured with freshly isolated CD45RA⁺CD45RO⁻CD4⁺ T_H cells. (A) Percentage of naive CD4⁺ T cells accumulating IFN- γ after 10 days of coculture and stimulation with PMA/ionomycin for 4 h as analyzed by flow cytometry and gated on CD4⁺CD3⁺ T cells. Representative data of 5 independent experiments are shown. (B) Summary of CD45RA⁺CD45RO⁻CD4⁺ T cells accumulating intracellular IFN- γ of 5 different donors.

Besides cytokine production, the other major NK cell function is cytotoxicity. To study the effect of NK-DC interaction on NK cell killing capacity, we investigated NK cell-mediated lysis of the Burkitt's lymphoma cell line Raji after 24 h incubation with DC supernatant. Raji cells are insensitive for lysis by naïve NK cells, however if NK cells are preactivated they become susceptible for NK cell-mediated cytotoxicity. Therefore IL-2 activated NK cells were taken along as positive control in the cytotoxicity assay. Only NK cells incubated with the supernatant of IFN- γ /FMKp matured DCs showed an increased cytotoxic activity, whereas NK cells incubated with the supernatant of TNF- α /PGE2 matured DCs did not (Figure 5A). Notably, in neither DC supernatant IL-2 was detected (data not shown), ruling out IL-2 as causative factor for augmentation of cytotoxicity. Another possible candidate, which has been shown to induce NK cell cytotoxicity is IL-12 (31). Therefore IL-12p70 was neutralized, which dose-dependently reduced the specific lysis of Raji cells to the level observed after co-incubation with unstimulated NK cells (Figure 5B-C). IL-15, presented *in trans* by IFN- γ /FMKp matured DCs, is another candidate cytokine responsible for

augmentation of NK cell mediated lysis. However, blocking of this cytokine in the supernatant of DCs, did not result in decreased killing capacity of NK cells (data not shown).

In conclusion, DCs triggered with bacterial fragments are able to induce IL-12/IL-18 mediated NK cell activation, resulting in the production of IFN- γ and T_H1 polarization. Additionally, the IL-12 produced by these DCs is also responsible for augmentation of NK cell cytotoxicity.

Figure 5. NK-DC interaction augments NK cell cytotoxicity.

Freshly isolated human NK cells and undiluted, filtered supernatants of TNF- α /PGE2 and IFN- γ /FMKp matured DCs were used for induction of NK cell cytotoxicity. NK cell cytotoxicity toward Raji cells was assessed by a flow cytometry-based kill assay. IL-2 activated NK cells represent a positive control and freshly isolated NK cells a negative control in inducing Raji cell lysis. (A) Data represent means $\pm$ SEM of triplicate wells. Data shown are representative of 6 independent experiments. (B) Cytotoxicity after neutralization of IL-12 in the supernatant of IFN- γ /FMKp matured DCs. Data represent means $\pm$ SEM of triplicate wells. Data shown are representative of 3 independent experiments. (C) Dose-dependent decrease of cytotoxicity after neutralization of IL-12 in the supernatant of IFN- γ /FMKp matured DCs in a effector-to-target ratio of 20:1. Columns, Mean of 3 different donors. bars, SEM. *, $P < 0.05$; **, $P < 0.01$.

Discussion

Under steady state conditions, NK cells do not home to tissues and lymph nodes. However, during inflammation it has been demonstrated that NK cells are able to migrate into inflamed tissue and draining lymph nodes (7, 25). At present it remains unclear what drives human NK cell migration *in vivo*. Primary immune responses to bacterial fragments (FMKP) induce DCs to produce high amounts of chemokines (Figure 1B) and we demonstrate that these DCs are able to recruit NK cells in a CCR5-dependent manner. It can be hypothesized that chemokines produced by DCs, triggered with bacterial fragments, are responsible for NK cell recruitment to inflamed tissue and into the inflammatory lymph node. The ability of human DCs to recruit NK cells is consistent with previous studies that report on CCL5 and CXCL10 production and NK cell recruitment by DCs either infected with *Mycobacterium Tuberculosis* (32), stimulated by oncolytic reovirus (33) or matured with IFN- α /IFN- γ /TNF- α /IL-1 β /poly-(I:C). Blocking of CXCR3 did not affect NK cell migration to IFN- γ /FMKp matured DCs, therefore our data suggest that CXCR3- and CCR5-dependent NK cell recruitment are two different mechanisms by which NK cells can be attracted by DCs. Both chemokine receptors CXCR3 and CCR5 are expressed by subpopulations of resting and activated NK cells and have been proven essential for NK cell migration to various tissues in response to pro-inflammatory stimuli (25, 28, 34). Which NK cell-recruitment mechanism is utilized seems to depend on the agents used in DC maturation and lack of NK cell recruitment by TNF- α /PGE2 matured DCs suggests that TLR-ligands or other PRR-triggering agents are necessary triggers for DCs to recruit NK cells. Triggering of different TLRs could explain the difference in chemokines dominant in NK cell recruitment and suggest that different infectious agents can induce different DC-chemokine profiles. This is supported by our data on differences in chemokine secretion between DCs triggered with TLR2 and TLR4 agonists, resulting in differential NK cell recruitment. Even though the current data indicate that TLR4 signaling induces DCs to secrete CCL5, it remains to be established whether this is the only pathway inducing production of this chemokine. IFN- γ /FMKp matured DCs produce 4-fold higher amounts of CCL5 than LPS/IFN- γ matured DCs which could be due to differences in the concentration of the TLR-triggering agents, since the exact concentrations of LPS and

OmpA in FMKp is unknown. Furthermore, at present it remains unclear whether FMKp also contains other PRR ligands responsible for higher chemokine secretion. A second line of evidence supporting the fact that different TLR triggers induce different DC chemokine profiles is illustrated in mouse studies on differential NK and T cell activation and recruitment by differently matured DCs (35, 36). Notably, the CXCR3- and CCR5-dependent mechanisms by which DCs attract NK cells are not mutually exclusive, as it has been demonstrated that upon poly (I:C) triggering, intrasplenic NK cell trafficking depends on synergistic actions of CXCR3 and CCR5 (34).

It can be envisioned that DCs, displaying a favorable chemokine profile, are able to recruit immune effector cells including NK cells, T_H1 cells and CTLs and thereby creating a peripheral, tertiary lymph node (37). Next to this peripheral NK cell recruitment, DCs could facilitate NK cell migration into the lymph nodes. This has been demonstrated in a mouse model by LPS-matured DCs, which recruit NK cells into the draining lymph nodes (3). Since IFN- γ /FMKp matured DCs are active producers of CCR7, CXCR3 and CCR5 ligands, it can be anticipated that they attract NK cells during their arrival in draining lymph nodes by virtue of different mechanisms and our *in vitro* data demonstrate that CCR5-dependent recruitment is the most potent for these DCs.

In addition to the CCR5-dependent NK cell recruitment, we show that DCs triggered by bacterial fragments have the ability to induce CCR7 expression on a subset of NK cells that increases their migratory responsiveness to CCL19. This represents a second mechanism of DC-induced NK cell homing into inflammatory lymph nodes. The factors inducing CCR7 expression in our system have not been elucidated. Similar results have recently been obtained by Marcenaro et al., who showed an induction of CCR7 after co-incubating NK cells and DCs in an allogeneic setting (38). We, however, observed CCR7 induction in both allogeneic as well as in autologous settings, indicating that multiple mechanisms may account for CCR7 induction. In line with findings of Mailliard *et al.*, who demonstrated (6), that IL-18 induces CCR7 expression on the same CD56^{dim}CD16⁺ NK cell subset as the IL-18 producing, IFN- γ /FMKp matured DCs (Supporting information Figure 5). Therefore IL-18 could be one of the DC-dependent factors responsible for the CCR7 induction. In addition, the partial contact-dependency may also point to IL-18, because it has been shown that

DC-derived IL-18 is polarized and secreted in the immunologic synapse inducing local, high IL-18 concentrations (39).

Besides their NK cell-attracting capacity, DCs matured with bacterial fragments also meet the prerequisites for NK cell activation. Using blocking antibodies, we demonstrated that both DC-derived IL-12 and IL-18 are necessary for IFN- γ secretion by a subpopulation of NK cells. Addition of rIL-12 to the supernatant of TNF- α /PGE2 matured DCs did not result in NK cell IFN- γ production (data not shown), which indicates that both IL-12 and IL-18 are required to induce IFN- γ secretion, supporting the two-signal requirement mechanism described by Mailliard *et al.* (6). Notably, the IFN- γ producing NK cell population contained both NK cells with an intermediate and with a high expression of CD56, which is remarkable since the CD56^{bright} subpopulation has been described as the cytokine producing population after NK-DC interaction (40). Upon physical contact with DCs, NK cells produce even higher amounts of IFN- γ (Supporting Information Figure 4), indicating that predominantly soluble factors, with minor contribution of contact-dependent factors, mediated IFN- γ secretion by NK cells. This phenomenon was observed in both autologous, as well as Killer cell Immunoglobulin-like Receptor- (KIR) mismatched NK-DC co-cultures. Ligation of NK cell receptors could be responsible for this contact-dependency (15), however, the formation of an immunological synapse could also be an important factor (41). Next to IL-18, other possible candidates are MICA/B (15) and *in trans* presented IL-15 (15, 30). The functional consequence of NK cell activation by IFN- γ /FMKp matured DCs appeared to be dual. On the one hand, NK cells produce sufficient cytokines to increase T_H1 polarization that was shown previously to be completely dependent on NK cell-derived IFN- γ (29). On the other hand, we demonstrated that IL-12 produced by IFN- γ /FMKp matured DCs also augments NK cell cytotoxicity toward the Raji cell line. This augmentation has been documented after contact of NK cells with some bacteria or by NK-DC crosstalk during bacterial infection (11, 42).

In conclusion, based on our *in vitro* data, it can be hypothesized that *in vivo* a *K. pneumonia* infection is responsible for induction of innate immune responses characterized by DC-dependent NK cell migration, facilitating NK-DC interactions both in the periphery as well as in lymphoid tissue. The molecular program triggered by FMKp and IFN- γ does not only permit DCs to recruit NK cells, but also induces the prerequisites

necessary to activate NK cell cytotoxicity and NK cell helper function for T_H1 polarization and possibly subsequent CTL induction. DC vaccination studies for the treatment of cancer have revealed that NK cell activation is of importance for sustained antitumor responses (43). Since the examined DC maturation cocktails are clinically relevant DC-preparations used in DC-based vaccination studies, unraveling the *in vitro* mechanisms of NK-DC interaction is of importance for the development of therapeutic DC vaccination and DC-targeting strategies for cancer and infectious diseases.

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Supporting Information

Figure 1. Surface phenotype of DCs matured by TNF- α /PGE2 or IFN- γ /FMKp.

DCs differentiated for 7 days in the presence of IL-4/GM-CSF and matured for 24 h with TNF- α /PGE2 or IFN- γ /FMKp (DCs were washed after 6 h maturation and were further matured for 18 h in AIM-V[®] medium without maturation stimuli) were used for flow cytometric comparison of surface expression of HLA-molecules and costimulatory markers. Filled areas represent surface expression on immDCs, bold lines on TNF- α /PGE2 matured DCs and dotted lines on IFN- γ /FMKp matured DCs. Representative data out of 4 different donors are shown.

Figure 2. NK cell migration towards a CCL19 gradient.

Number of freshly isolated NK cells (10^5 cells) migrated towards different CCL19 (0 ng/ml, 62,5 ng/ml, 125 ng/ml and 250 ng/ml) gradients in a Transwell[®] assay in different time periods (4, 8 and 16 h). Columns, Mean values; bars, SEM. *, $P < 0.05$; **, $P < 0.01$.

Figure 3. NK cell activation phenotype.

Phenotype after 24 h coculture with TNF- α /PGE2 or IFN- γ /FMKp matured DCs as detected by flow cytometry. (A) CD25 and (B) CD69 expression gated on CD56⁺NKp46⁺CD3⁻ NK cells. Filled histogram NK cell coculture with TNF- α /PGE2 and dotted line IFN- γ /FMKp matured DCs. Representative data of 3 different donors.

Figure 4. Contact-dependent IFN- γ secretion.

Quantitative comparison of IFN- γ production by NK cells (10^5 cells/well) cultured for 16 h NK in either the upper well of a 0,4 μ m Transwell[®] plate, separated from DCs (only soluble factors can induce NK cell activation) or in the lower well where they were cocultured with DCs (2×10^5 cells/well), as measured by ELISA. *Columns*, Mean values of 9 different donors, *bars*, SEM. *, $P < 0.05$; **, $P < 0.01$.

Figure 5. IL-18 induced CCR7 expression by NK cells.

CCR7 expression after 24 h coculture with IFN- γ /FMKp matured DCs or IL-18 as detected by flow cytometry and gated on CD56⁺NKp46⁺CD3⁻ NK cells.

Chapter

5

Inflammation restraining effects of Prostaglandin E2 on NK-DC interaction are imprinted during dendritic cell maturation

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Abstract

Among prostaglandins (PG), PGE2 is abundantly expressed in various malignancies and is probably one of many factors promoting tumor growth by inhibiting tumor immune-surveillance. In the current study, we report on a novel mechanism by which PGE2 inhibits *in vitro* NK-DC crosstalk and thereby innate and adaptive immune responses via its effect on NK-DC crosstalk. The presence of PGE2 during IFN- γ /FMKp dendritic cell (DC) maturation inhibits the production of chemokines (CCL5, CCL19 and CXCL10) and cytokines (IL-12 and IL-18), which is cAMP-dependent and imprinted during DC maturation. As a consequence, these DCs fail to attract natural killer (NK) cells and show a decreased capacity to trigger NK cell IFN- γ production, which in turn leads to reduced T_H1 polarization. In addition, the presence of PGE2 during DC maturation impairs DC-mediated augmentation of NK cell cytotoxicity. Opposed to their inhibitory effects on peripheral blood-derived NK cells, PGE2 matured DCs induce IL-22 secretion of inflammation constraining NKp44⁺ NK cells present in mucosa-associated lymphoid tissue. The inhibition of NK-DC interaction is a novel regulatory property of PGE2 that is of possible relevance in dampening immune responses *in vivo*.

Introduction

Prostaglandins (PG) are potent immune modulators, which are produced during inflammation after the conversion of arachidonic acid by cyclooxygenase (COX)(1). Furthermore, PG are also abundantly produced by various types of tumors (2). COX2 expression, which is correlated with a poor prognosis, is induced in a variety of human premalignant and malignant tumors, including solid tumors as well as hematological malignancies (3-6). Several lines of evidence demonstrate that COX2-derived PGs are involved in the promotion of tumor growth by regulation of cancer cell proliferation, apoptosis, migration and invasion (7-11). PGs are also produced by tumor-surrounding cells, creating a tumor-supporting environment by enhancing angiogenesis and inhibiting tumor immune surveillance (2, 11-14).

Of all prostaglandins, PGE2 has a pivotal role in tumor immunosuppression. It has been hypothesized that this effect is caused by induction of a permanent state of inflammation (2), resulting in phenotypic and functional changes of T helper (T_H) cells, cytotoxic T (CTL) cells, dendritic cells (DC), natural killer (NK) cells and myeloid-derived suppressor cells (MDSC) (12). PGE2 has been shown to deviate T_H cell skewing from an antitumor T_H1 response toward a T_H2/T_H17 response by direct binding to these cells (15-17). Additionally, PGE2 is responsible for shifting the balance of IL-12/IL-23 production by DCs toward IL-23, which is a very potent cytokine responsible for T_H17 expansion and survival (18, 19). As a consequence, less IL-12 and other pro-inflammatory cytokines are produced, thus inhibiting T_H1 polarization (17, 20). PGE2 also decreases the cytotoxic capacity of CTLs directly by inducing the expression of inhibitory receptors on CTLs (21) and indirectly by inhibiting DC maturation and antigen presentation (22, 23). Moreover, tumor-associated PGE2 has been reported to be responsible for the preferential attraction and induction of regulatory T (Treg) cells, creating an immune-regulatory microenvironment (24, 25).

Next to the modulating effects of PGE2 on the effector mechanisms of the adaptive immune response, PGE2 also has an effect on the innate immune response. NK cells, which are implicated in the innate defense against virally infected and malignantly transformed cells, have been reported to be affected by PGE2. It has been shown that

PGE2 suppresses proliferation, cytokine secretion, migration and NK cell-mediated cytotoxicity both *in vivo* and *in vitro* (26-30).

Even though PGE2 has these direct effects on NK cells, it remains to be established whether PGE2 also has an effect on NK-DC crosstalk. We and others have previously reported on the importance of NK-DC crosstalk in modulating adaptive immune responses (29, 31, 32). This crosstalk is characterized by DC-dependent recruitment of NK cells and IFN- γ production by NK cells, of which the latter contributes to strong T_H1 polarization (32). Additionally, DCs augment NK cell-mediated cytotoxicity of tumor cells. The DC-derived cytokines IL-12, IL-18 and IL-15 have been implicated in NK cell activation and IL-15 is also responsible for proliferation and survival of NK cells (32, 33).

In the current study, we investigated how PGE2 influences NK-DC interactions. We identified the effects of PGE2 signaling on the production of key chemokines and cytokines responsible for NK cell interaction with DCs matured with IFN- γ and a membrane fraction of *Klebsiella pneumoniae* (FMKp). We show that PGE2 not only has a direct effect on NK cells, but also indirectly alters DC-mediated, NK cell effector functions.

Materials and methods

Cell isolation

Mononuclear cells from peripheral blood of healthy donors were isolated by density gradient separation using lymphoprep (Axis-Shield). NK cells and naïve CD4⁺ T cells were negatively selected by immunomagnetic cell separation (Miltenyi Biotec GmbH). The purity of isolated populations exceeded 95% as determined by flow cytometry.

Human tonsils were obtained from the Department of Otorhinolaryngology-Head & Neck Surgery, Maastricht University Medical Center. NK cells were isolated to a purity of 70-80% as described by Cella et al. (34).

Generation of DCs

DCs were prepared from peripheral blood-derived monocytes, isolated by density centrifugation and adherence as previously described (35). In addition, highly purified

monocyte fractions obtained by elutriation of leukapheresis products were used (purity >95%). Written informed consent was obtained from all leukapheresis donors in accordance with the Declaration of Helsinki. For every experiment, DCs from both isolation methods were used to avoid the effect of contaminating NK cells (<5% in density centrifugation isolated monocytes) on the results. Differentiation of monocytes was induced during 6 days of culture in AIM-V[®] medium (Gibco Life Technologies) containing 2000 U/ml IL-4 (Strathmann Biotech) and 400 U/ml GM-CSF (Berlex). DCs were matured for 6 h in AIM-V[®] medium containing 500 U/ml IFN- γ (Strathmann Biotech), 1 μ g/ml FMKp (Pierre Fabre) and different concentrations of PGE2 (Sigma-Aldrich). Where indicated, 10 μ M butaprost (EP2 agonist), 35 μ M misoprostol (EP2/EP3/EP4 agonist), 10 μ M sulprostone (EP1/EP3 agonist; Cayman Chemical), or 100 μ M of dibutyryl-cAMP (Alexis Biochemicals) were added. When selective EP-receptor agonists were compared, 10 μ M PGE2 was used.

Flow cytometry

All antibodies for flow cytometry were obtained from BD Biosciences, except for CCR7, 2B4, CXCR3, CCR5, NKp80, NKp44, IL-22 (R&D Systems), NKp30 (BioLegend) and NKG2A, NKG2D, CD158A, CD158B, CD158D, CD158E (Miltenyi Biotec GmbH). Cells were incubated with antibodies at proper dilutions for 30 minutes at RT. Analyses were performed on a FACSCanto (BD Biosciences) and analyzed with BD CellQuest[™] Pro Software (BD Biosciences), WinMDI (Joe Trotter, <http://facs.scripps.edu/>) or FlowJo (Treestar, Ashland, OR) software.

Migration assay

Migration of NK cells was analyzed as described previously (32). To analyze the effect of CCL5 reconstitution on NK cell recruitment, supernatant of IFN- γ /FMKp DCs matured in the presence of PGE2 was supplemented with 50 ng/ml CCL5. Migration of DC was analyzed as described previously (35).

Cytokine and chemokine secretion by DCs

Quantification of IL-18, IL-12, IL-23, CCL5, CXCL10 and CCL19 in 24 h maturation supernatants was performed using ELISA (MBL International Corporation and R&D Systems) according to manufacturer's instructions.

Cytokine production by NK cells

NK cell cells were either cultured in the presence of PGE2 and stimulated with 2,5 µg/ml phorbol 12-myristate 13-acetate (PMA) and 2 µg/ml ionomycin or with supernatant of DCs matured with different concentrations of PGE2. To analyze the effect of CCL5 on NK cell recruitment, supernatant of IFN-γ/FMKp DCs matured in the presence of PGE2 were supplemented with 2 ng/ml IL-12 and/or 100 pg/ml IL-18. Quantification of IFN-γ production was performed after 16 h of stimulation using an IFN-γ ELISA kit (R&D systems).

To evaluate IL-22 production by NK cells obtained from tonsils, NK cells were incubated with DCs in 1:2 ratio. Flow cytometric analysis of IL-22 secreting NK cells was performed as described by Cella et al. (34).

T_H cell priming

In a 96-well plate, IFN-γ/FMKp matured DCs (4×10^4 cells/well) were coated with 1 ng/ml Staphylococcal Enterotoxin B (SEB, Sigma-Aldrich) for 1 h. SEB-coated DCs, were washed and placed in culture with CD45RA⁺/RO⁻CD4⁺ T cells (1×10^5 cells/well). Every other day stimulation medium was replaced with supernatant of NK-DC cultures. In these NK-DC cultures, IFN-γ/FMKp matured DCs or IFN-γ/FMKp + 1000 ng/ml PGE2 matured DCs (2×10^5 cells/well) were cultured with/without NK cells (1×10^5 cells/well). At day 10 the expanded T_H cells were washed, plated in 96-well plates (10^5 cells/well), and stimulated with PMA/ionomycin (BD Biosciences). After 4 h, cells were harvested and surface and intracellular staining was performed as described (32).

NK cell cytotoxicity assay

For NK cell-mediated lysis of K562, unstimulated NK cells were cocultured with K562 cells for 4 h in the presence of different concentrations of PGE2. For NK cell-mediated lysis of

Raji, NK cells were preactivated for 18 h by either supernatant of IFN- γ /FMKp DCs matured in the presence of different concentrations of PGE2, medium alone or medium containing 1000 U/ml IL-2 (Proleukin, Chiron Benelux BV). Target cells were labeled with 3'-diiododecylthiopyranine (DiO) according to the manufacturer's instructions (Sigma) and 2×10^4 target cells were incubated with pre-activated NK cells at various effector:target (E:T) ratios for 12 h, each ratio in triplicate. Percentages of killed target cells (PI⁺DiO⁺) were determined by flow cytometry. Percentages specific lysis were calculated as follows (36):

$$\frac{\% \text{ total target cell death} - \% \text{ spontaneous target cell death (PI}^+) }{\% \text{ vital cells (\% PI- cells not incubated with effector cells)}} \times 100\%$$

Statistics

Statistical significance of differences between experimental samples was determined using Student's t test for paired samples, ANOVA or Wilcoxon signed-rank test. Significance was accepted at the $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) levels. Data were analyzed using Prism software version 5.00 (GraphPad Software).

Results

PGE2 directly inhibits NK cell cytokine production and NK cell-mediated cytotoxicity

We investigated whether PGE2 affects the expression of inhibitory and activating receptors on the NK cell surface. To this end, freshly isolated NK cells were cultured for 24 h in the presence or absence of PGE2. As shown in Figure 1A, only expression of NKG2D (NK cells: mean MFI $60,3 \pm 16$ and NK cells + PGE2: mean MFI $34,8 \pm 9,7$) and 2B4 (NK cells: mean MFI $103 \pm 4,5$ and NK cells + PGE2: mean MFI $70,8 \pm 2,9$) is significantly ($P < 0,05$) reduced in six different donors (Wilcoxon signed rank test) due to PGE2 treatment. The expression of the other activating and inhibitory NK cell surface markers, including specific Killer Immunoglobulin Receptors (KIRs), did not change. Additionally, expression of the chemokine receptors CCR5, CXCR3 and CCR7 was evaluated, since these chemokine receptors are implicated in NK cell migration (29, 31). PGE2, however had no effect on the expression of these receptors. As PGE2 has been shown to enhance IL-23 receptor expression by T_H17 cells (15), we evaluated if PGE2 had the same effect on NK cells. Flow cytometric analysis showed that IL-23 receptor expression on NK cells was not altered. The effect of PGE2 on surface expression of the activating receptors 2B4 and NKG2D suggests that PGE2 has an inhibitory impact on NK cell effector functions. To evaluate this impact, cytotoxicity assays were performed in either the presence or absence of PGE2. As target cells, K562 cells were used, since these cells fail to express HLA class I and are therefore efficiently killed by NK cells. A dose-dependent decrease in cytolytic activity was observed when PGE2 was present during the cytotoxicity assay (Figure 1B). Similar data have been reported previously (37). To evaluate the effect of PGE2 on IFN- γ production, a second NK cell effector function, NK cells were stimulated with PMA and ionomycin in the presence or absence of different concentrations of PGE2 and 16 h supernatant was evaluated by ELISA for the presence of IFN- γ production. PGE2 dose-dependently inhibited IFN- γ secretion (Figure 1C).

Taken together these results show that PGE2 has a direct effect on NK cells, as it downregulates the expression of the NK cell-activating receptors 2B4 and NKG2A and it dose-dependently inhibits NK cell-mediated cytotoxicity and IFN- γ secretion.

Figure 1. Direct effect of PGE2 on NK cell phenotype, cytotoxicity and cytokine secretion.

Freshly isolated NK cells were cultured for 24 h in the presence of PGE2. (A) Phenotypic analysis of NK surface receptor expression. Filled histograms, NK cells without PGE2, Bold lines, NK cells with 1000 ng/ml PGE2. Representative data of one out of 6 different donors. (B) Flow cytometry-based NK cell cytotoxicity assay against K562 by NK cells cultured with different concentrations of PGE2. Data shown are triplicates of one representative experiment out of 4 different experiments. (C) IFN- γ production by NK cells cultured with different concentrations of PGE2 and stimulated for 16 h with PMA and ionomycin, as evaluated by ELISA. Results are presented as mean plus SEM and are obtained from 3 different donors. * $P < 0.05$.

Effect of PGE2 on DC-derived chemokines is imprinted during maturation and inhibits NK cell recruitment

NK cells can be recruited by mature DCs ultimately leading to NK cell activation (31, 32, 38). This recruitment depends on DC-derived chemokines of which CCL5, CXCL10 and CCL19 are the most important. To study whether PGE2 treatment of DCs influences NK cell recruitment due to effects on the DC chemokine profile, we evaluated CCL19, CXCL10 and CCL5 secretion by DCs after maturation in the presence or absence of PGE2. To this end, monocyte-derived DCs were matured with a membrane fraction of *Klebsiella pneumoniae*

(FMKp) and IFN- γ as previously described (35). DCs were matured for 6 h with IFN- γ /FMKp and different concentrations of PGE2. After washing, to remove all maturation factors, DCs were incubated for another 18 h in medium only (total maturation of 24 h). Effectiveness of DC washing was evaluated by detection of PGE2 by ELISA. In none of the samples PGE2 was found (data not shown), indicating that IFN- γ /FMKp matured DCs do not produce PGE2. Addition of PGE2 during DC maturation did not affect upregulation of HLA-DR and co-stimulatory molecules (CD80, CD83, CD86 and CD40), suggesting that addition of PGE2 to the maturation cocktail did not hinder the development of mature DCs (Supporting information Figure 1A). However, PGE2 did induce a significant increase in surface expression of CCR7 (Supporting information Figure 1B).

Increased CCR7 expression by TLR-triggered DCs cultured in the presence of PGE2 has been described previously (39). IFN- γ /FMKp matured DCs also showed a dose-dependent increase of CCR7 expression, which indeed resulted in functionally increased responsiveness to the lymph-node associated chemokine CCL19 (Supporting information Figure 1C).

Chemokine (CXCL10, CCL19 and CCL5) production was evaluated by culturing DCs for 14 h in the presence of different PGE2 concentrations and quantified by ELISA (Figure 2A). Production of these chemokines significantly decreased with increasing concentration of PGE2. Since CCR5 has been identified to be the most important chemokine receptor responsible for NK cell recruitment by IFN- γ /FMKp matured DCs (32), the negative effect of PGE2 on CCL5 production suggested a decrease in the capacity of PGE2 matured DCs to recruit NK cells. To test this hypothesis, migration assays were performed. Freshly isolated NK cells were allowed to migrate during 1,5 h to supernatant of DCs matured in the presence of different concentrations of PGE2. NK cell recruitment was significantly decreased when increasing doses of PGE2 were added during DC maturation (Figure 2B). Since DCs were extensively washed after 6 h of maturation, DC supernatant did not contain any PGE2 anymore, therefore NK cells were not directly exposed to PGE2. This indicates that the negative effect on NK cell migration does not depend on an effect of PGE2 on NK cell chemokine receptors, which has previously been described (29). After supplementing the supernatant of PGE2 matured DCs with CCL5, NK cell recruitment was

restored, indicating that indeed the effect of PGE2 on CCL5 production was responsible for decreased NK cell recruitment (Figure 2C).

Figure 2. PGE2 inhibits DC-derived CXCL10, CCL5 and CCL19 secretion.

Monocyte-derived DCs were matured with IFN-γ/FMKp in the presence of different concentrations of PGE2. After 6 h maturation, DCs were washed to remove the stimulation medium, and matured for an additional 18 h in AIM-V® medium only. (A) Quantitative comparison of CXCL10, CCL5 and CCL19 production as measured by ELISA. Results shown are the mean plus SEM of combined data of at least 5 different donors. (B) Percentage of NK cells migrated in 1.5 h toward cell free supernatant of IFN-γ/FMKp DCs matured in the presence of different concentrations of PGE2. Data were calculated as percentage of migrated NK cells. Results presented are the mean plus SEM of combined data of 11 different donors. (C) Percentage of NK cells migrated in 1.5 h toward cell free supernatant of IFN-γ/FMKp DCs matured in the presence of PGE2 and supplemented with 50 ng/ml CCL5. Data were calculated as percentage of migrated NK cells, normalized to NK cell migration toward IFN-γ/FMKp DCs matured without PGE2. Results presented are the mean plus SEM of combined data of 4 different donors.

DCs have been shown to express all four different EP receptors (40). To evaluate which EP receptors mediate the effect of PGE₂ on chemokine production by DCs, they were stimulated with specific agonists that preferentially bind to one or more EP receptors (Figure 2D). Stimulation with butaprost and misoprostol reduced CXCL10 and misoprostol also inhibited CCL5 secretion significantly, whereas sulprostone did not alter secretion of these chemokines. This indicates that mainly EP2 and EP4 signaling are responsible for the effect of PGE₂ on the reduced secretion of CXCL10 and CCL5. In contrast, CCL19 production was equally affected by mistoprostol and butaprost, demonstrating that EP2 is the most important EP receptor involved in the inhibition of CCL19 production. The addition of the intracellular cAMP analogue dibutyryl-cAMP mimicked the effect of PGE₂ for production of all three chemokines, suggesting that the effect of PGE₂ was dependent on cAMP signaling.

To evaluate whether the effect of PGE₂ on chemokine production by DCs is imprinted during maturation or whether PGE₂ can also exert its suppressive effect after initiation of maturation, chemokine production of DCs was analyzed when PGE₂ was added during or after the first 6 h of DC maturation. After 24 h maturation in total, chemokine production was evaluated (Figure 2E). A decrease in CXCL10 and CCL5 secretion could only be detected when PGE₂ was present during the first 6 h of maturation. CCL19 production was also affected by PGE₂ after the 6 h maturation period, however the effect was less pronounced as compared to the effect of immediate addition of PGE₂.

These results indicate that the negative effect of PGE₂ on production of DC-derived CXCL10, CCL5 and CCL19 is imprinted during maturation and depends on EP2/EP4-mediated cAMP signaling. In addition, we show that the NK cell recruiting capacity of DCs is reduced due to the influence of PGE₂.

Figure 2. PGE2 inhibits DC-derive CXCL10, CCL5 and CCL19 secretion (continued).

(D) Quantitative comparison of CXCL10, CCL5 and CCL19 production after stimulation with different EP receptor agonists and cAMP as measured by ELISA. Results shown are the mean plus SEM of combined data of at least 4 different donors. (E) Quantitative comparison of CXCL10, CCL5 and CCL19 production after stimulation with PGE2 either during or after DC maturation as measured by ELISA. Results shown are the mean plus SEM of combined data of at least 4 different donors. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Effect of PGE2 on DC-derived IL-12/IL-18 production is imprinted during maturation and inhibits NK cell IFN- γ secretion

We previously showed that production of IL-12 and IL-18 by IFN- γ /FMKp matured DCs is needed to induce NK cell activation (32). To evaluate the effect of PGE2 on NK cell activation, production of these cytokines by IFN- γ /FMKp DCs stimulated with PGE2 was determined in 24 h DC-supernatant. As described previously, TLR-triggered DCs produce less IL-12 when matured in the presence of PGE2 (17, 39). We show here that PGE2 also has this effect on IL-12 production by IFN- γ /FMKp matured DCs and, additionally, PGE2 triggering results in less IL-18 secretion (Figure 3A). Moreover, PGE2 exerted its effect on the secretion of these cytokines only when it was present during DC maturation, as evidenced by lack of inhibition of IL-12 and IL-18 production when PGE2 was added after the 6 h DC-maturation period (Figure 3B).

Figure 3. PGE2 inhibits DC-derived NK cell activation.

Monocyte-derived DCs were matured with IFN- γ /FMKp in the presence of different concentrations of PGE2. After 6 h maturation, DCs were washed to remove the stimulation medium, and matured for an additional 18 h in AIM-V[®] medium only. (A) Quantitative comparison of IL-12 and IL-18 production as measured by ELISA. Results shown are the mean of combined data of at least 4 different donors plus SEM (IL-12, n=16; IL-18, n=4). (B) Quantitative comparison of IL-12 and IL-18 production after stimulation with PGE2 either during or after 6 h DC maturation as measured by ELISA. Results shown are the mean of combined data of at least 3 different donors plus SEM.

NK cell activation, evaluated by IFN- γ production of NK cells cultured for 16 h in DC supernatant, was significantly reduced when DCs were matured in the presence of PGE2 (Figure 3C). NK cell activation was completely restored after addition of IL-12 to the supernatant of IFN- γ /FMKp DCs matured in the presence of PGE2, indicating that the effect of PGE2 on DC-mediated NK cell activation mainly depends on the inhibition of IL-12 secretion (Figure 3D). Although the decrease in DC-mediated NK cell activation is caused by the decrease in IL-12 secretion, these data do not exclude that other factors might contribute. One candidate cytokine produced by TLR-triggered DCs in the presence of PGE2 is IL-23, a cytokine implicated in the induction of T_H17 responses (19). Also IFN- γ /FMKp matured DCs produce IL-23, which is enhanced when DCs are matured in the

presence of PGE2 (Figure 3E), albeit with an apparent optimum at 50 ng/ml PGE2. Addition of IL-23 to DC supernatant during NK cell activation did not affect IFN- γ production by NK cells (data not shown). These data indicate that PGE2 added to the maturation of DCs is not only responsible for decreased production of the NK-activating cytokines IL-12 and IL-18, but also enhances IL-23 secretion.

Figure 3. PGE2 inhibits DC-derived NK cell activation (continued).

(C) NK cell IFN- γ production, as measured by ELISA, after incubation for 16 h with DC supernatant. Results presented are the mean plus SEM of combined data of 11 different donors. (D) NK cell IFN- γ production, as measured by ELISA, after incubation for 16 h with DC supernatant, or DC supernatant supplemented with 2ng/ml IL-12, 100pg/ml IL-18 or both. Results presented are the mean plus SEM of combined data of 6 different donors. (E) Quantitative comparison of IL-23 production as measured by ELISA. Results shown are the mean plus SEM of combined data of 4 different donors. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Presence of PGE2 during DC maturation stimulates IL-22 production by NKp44⁺ NK cells isolated from human tonsils

Recent studies showed that in mucosa-associated lymphoid tissue, a NK cell subset with inflammation restraining properties resides. This NKp44⁺ subset produces IL-22, IL-26, and LIF in response to IL-23 or after co-culture with TLR-matured DCs and are not proficient at typical NK cell functions such as cytotoxicity and IFN- γ secretion (34). In contrast to conventional NK cells, that secrete cytokines in response to IL-12, this NK cell subpopulation produces IL-22 after IL-23 triggering and, to a lesser extent, after triggering with IL-6 and IL-15.(34) Furthermore, these NKp44⁺ NK cells express the transcription factor ROR γ T and therefore show functional and phenotypic similarity to T_H17 cells (41, 42). Given the positive effect of PGE2 matured DCs on the induction of T_H17 cells,(15) we hypothesized that the addition of PGE2 to IFN- γ /FMKp DC maturation may enhance activation of NKp44⁺ cells.

Figure 4. Effect of PGE2 matured DCs on IL-22 production by NK cells residing in the tonsil.

Monocyte-derived DCs were matured with IFN- γ /FMKp in the presence or absence of 50 ng/ml PGE2. After 6 h maturation, DCs were washed to remove the stimulation medium, and matured for an additional 18 h in AIM-V[®] medium only. (A) Percentage of NKp44⁺ NK cells isolated from tonsils, accumulating IL-22 after 16 h of coculture as analyzed by flow cytometry, gated on CD56⁺CD3⁻ cells. Representative data of one out of 6 independent experiments are shown. (B) NKp44⁺ NK cells accumulating intracellular IL-22 of 7 different donors. In 6 out of 7 donors there is an increase in % of IL-22 positive NK cells. Wilcoxon signed-rank test significance * $P < 0.05$.

To test this hypothesis, NK cells were isolated from human tonsils and cocultured for 16 h with DCs that were matured in the presence or absence of 50 ng/ml PGE2. Production of IL-22 by NKp44⁺ NK cells was evaluated by flow cytometry. There was a significant increase in percentage of NK cells accumulating IL-22 when NK cells were cocultured with PGE2-stimulated DCs as compared to cocultures with DCs matured in the absence of PGE2 (Figure 4A, B). Notably, the IL-22 producing NK cell subset expressed NKp44⁺ and did not accumulate IFN- γ .

Taken together, these data indicate that PGE2 is responsible for the induction of DCs that activate inflammation restraining NK cells.

Decreased activation of NK cells by PGE2-matured DC inhibits NK cell-dependent T_H1 polarization and augmentation of cytotoxicity

NK-derived IFN- γ has been implicated in the induction of T_H1 polarization (31, 43). Consequently, it can be anticipated that the decreased activation of NK cells by DCs matured in the presence of PGE2 results in decreased T_H1 polarization. To evaluate whether this hypothesis is true, DCs (washed after 6 h maturation) were matured with or without PGE2 and after 24 h NK cells were added. In this experiment we were only interested in the effect of NK cell-derived cytokines on T_H1 polarization and not in the previously reported negative effect of PGE2 matured DCs on T_H1 skewing. Therefore, DCs without PGE2 stimulation were coated with SEB and used in a T cell stimulation assay. To study the effect of NK cell-secreted cytokines, T cell stimulation medium was exchanged every other day by the supernatant of the NK-DC cocultures (Figure 5A). T_H1 polarization was detected by accumulation of the T_H1 cytokine IFN- γ (Figure 5B). Only T cells stimulated with medium of NK-DC cocultures, in which the DCs were not triggered with PGE2 during maturation, benefited from the T_H1 polarizing effect of NK cells, as more IFN- γ producing T cells were detected (Figure 5C).

Previously, we showed that IFN- γ /FMKp matured DCs are able to augment NK cell cytotoxicity, which is mediated by DC-derived IL-12 (32). These observations opened the possibility that PGE2 triggering of DCs could abrogate the beneficial effect on NK cell cytotoxicity. To evaluate this, killing assays against the NK-cell resistant Raji cells were performed. Raji cells are insensitive for lysis by naive NK cells, however if NK cells are pre-

activated, they become able to kill Raji cells. When DCs were matured with increasing concentrations of PGE2, NK cell-mediated lysis of target cells decreased (Figure 5D). These data indicate that PGE2 does not only inhibit NK cell cytotoxicity via direct action on NK cells (Figure 1B), but also indirectly affects augmentation of NK cell cytotoxicity via its effect on DC maturation. Taken together, these data show that PGE2 inhibits NK cell-mediated cytotoxicity via two independent mechanisms.

Figure 5. Effect of PGE2 matured DC on NK cell function.

(A) IFN- γ /FMKp matured DCs with or without 5 μ g/ml PGE2 were cultured for 24 h in the presence or absence of freshly isolated NK cells. IFN- γ /FMKp matured DCs were coated with Staphylococcal Enterotoxin B and cocultured with freshly isolated CD45RA $^{+}$ /RO $^{+}$ CD4 $^{+}$ T $_{H}$ cells, every other day stimulation medium was exchanged with supernatant from IFN- γ /FMKp matured DCs (with or without 5 μ g/ml PGE2) cultured with or without NK cells. (B) Percentage of naive CD4 $^{+}$ T cells accumulating IFN- γ after 10 days of coculture and stimulation with PMA/ionomycin for 4 h as analyzed by flow cytometry, gated on CD4 $^{+}$ CD3 $^{+}$ T cells. Representative data of one out of 3 independent experiments are shown.

Figure 5. Effect of PGE2 matured DC on NK cell function (continued).

(C) CD45RA⁺/RO-CD4⁺ T_H cells accumulating intracellular IFN- γ of 5 different donors. Results presented are the mean of combined data of 5 different donors plus SEM. (D) Freshly isolated human NK cells and undiluted, filtered supernatants of IFN- γ /FMKp DCs matured in the presence of different concentrations of PGE2 were used for induction of NK cell cytotoxicity. NK cell cytotoxicity toward Raji cells was assessed by a flow cytometry-based kill assay. Data represent means $\pm$ SEM of triplicate wells. Data shown are representative of 3 independent experiments. (E) Cytotoxicity toward Raji without, after preincubation and early incubation of NK cells with PGE2 and activation in DC supernatant. Data represent means plus SEM of triplicate wells of E:T ratio of 10:1. Data are representative of 3 independent experiments. * $P < 0.05$.

To determine whether activation of NK cells by NK-DC crosstalk could overcome the negative effect of PGE2 on NK cell mediated cytotoxicity, killing of Raji cells was tested with either NK cells pre-incubated with PGE2 (pre-incubation) before stimulation with DC supernatant or NK cells triggered with PGE2 during stimulation with DC supernatant (early incubation). Flow cytometric analysis showed that NK cells pre-incubated with PGE2 are able to kill Raji cells when activated by DCs (Figure 5E). When PGE2 was present during the NK cell activation kill of Raji cells was decreased. However, when NK cells are pre-activated by DCs, PGE2 had a minor inhibitory effect on NK cell cytotoxicity, indicating that the negative effect of PGE2 on NK cells is partly overcome.

In summary, these results show that PGE2 matured DCs inhibit NK-cell mediated T_H1 polarization and also suppress augmentation of NK cell killing capacity.

Discussion

COX2-derived PGE2 is abundantly expressed in various malignancies and is one of many factors that directly promote tumor growth by regulation of cancer cell proliferation, apoptosis, migration and invasion (7-11). Moreover, PGE2 plays a pivotal role in immunosuppression creating a tumor supportive environment by inhibiting tumor immune-surveillance. Increasing the knowledge about the mechanisms by which PGE2 mediates immunosuppression is crucial in understanding tumor formation and might lead to the development of new immunotherapeutic strategies for cancer.

In this paper, we report on a novel mechanism by which PGE2 inhibits NK cell effector functions. In addition to its inhibition of NK cell activation by direct binding to NK cells, PGE also inhibits crosstalk with IFN- γ /FMKp matured DCs. This is mediated via modulation of chemokine and cytokine secretion of IFN- γ /FMKp matured DCs, which is responsible for inhibition of NK cell recruitment and activation. We show that PGE2 has an inhibitory effect on the secretion of CCL5, CXCL10 and CCL19, which are implicated in NK cell migration (31, 38). Decreased production of DC-derived CCL19 (implicated in naïve T cell recruitment) and CCL5 due to PGE2 triggering has recently been described (22, 44). Interestingly, the effect of PGE2 on chemokine secretion is imprinted during DC maturation as we show that PGE2 stimulation after maturation does not influence chemokine secretion.

Additionally, our data evidence that NK cell activation is altered due to the effect of PGE2 on IFN- γ /FMKp DC-derived cytokine secretion. PGE2-induced changes in the IL-12/IL-23 balance of DCs have been described to account for skewing of T_H1 responses toward T_H2 / T_H17 responses (15, 16, 19). Here, we show that it is also responsible for decreased IFN- γ secretion by NK cells. In addition, PGE2 is responsible for decreased production of IL-18, which is besides IL-12 an important cytokine for NK cell activation. This is in apparent paradox, to the reported IL-18 mediated induction of PGE2 secretion in the synovial fluid

of osteoarthritis patients (45). Possibly, this represents a feed-back loop by which PGE2 constrains inflammation during a normal immune response. Mainly the decreased production of IL-12 by IFN- γ /FMKp matured DCs due to PGE2 accounts for the inhibition on IFN- γ secretion, since addition of IL-12 restores IFN- γ secretion by NK cells. However, our data do not preclude that other cytokines that have an inhibitory effect on NK cell activation are produced by DCs in response to PGE2 triggering. As a functional consequence of the DC-mediated effect of PGE2 on NK cell activation, NK cell-dependent T_H1 polarization and tumor cell lysis is inhibited. Interestingly, the reported effect of PGE2 on NK-DC interaction was not only demonstrated for monocyte-derived DCs, but also for blood-derived BDCA1⁺ DCs that were matured with IFN- γ /FMKp in the presence of PGE2 (Supporting Information Figure 2). Additionally, the effect of PGE2 could also be induced by tumor cell-derived PGE2 (Supporting Information Figure 3 A,B). Under different cell culture conditions the same tumor cells did not produce PGE2, but were still able, though less efficiently, to inhibit NK-DC crosstalk (Supporting Information Figure 3A,B). A possible candidate for this inhibition is TGF- β , which is possibly one of many immunosuppressive factors produced by this cell line (Supporting Information Figure 3C). Thereby these data illustrate the redundancy of different tumor-derived factors on NK-DC crosstalk, during different culture conditions. We anticipate that among the many mechanisms tumors have to escape immune surveillance, PGE2 is very potent to prevent NK-DC interactions because the application of COX-inhibitors in tumor bearing mice completely restores the reduced CCL5 and IL-12 production of myeloid cells (14, 44).

In relation to the enhanced production of IL-23 by PGE2 stimulated DCs, it is relevant to note that Cella et al. reported on an NK cell subset (NK-22 cells) that resides in mucosa-associated lymphoid tissue, expresses NKp44 and produces IL-22 rather than IFN- γ upon IL-23, IL-6 and IL-15 stimulation (34). Additionally, they showed that these NK cells produce IL-22, IL-26 and LIF after contact with IL-23 producing monocytes that were stimulated with LPS. This NK cell subset was suggested to contribute to mucosal homeostasis, since IL-22 induces production of the anti-inflammatory cytokine IL-10 by epithelial cells (34, 41, 46, 47). We show here that PGE2 is responsible for the maturation of DCs that are capable of inducing mucosal NKp44⁺, IL-22 secreting NK cells and not IFN- γ secreting peripheral NK cells. Given the fact that NKp44⁺ NK cells produce IL-22 in

response to IL-23, the increased IL-23 secretion of PGE2 matured IFN- γ /FMKp DCs could represent one of several mechanisms by which DCs induce IL-22 secretion of these NK cells.(34) In this light, it is interesting to note that IL-22 as well as tumor-derived PGE2 have the same immune-protective effects on epithelial cells, which is characterized by increased proliferation and decreased apoptosis (7). It can be hypothesized that the IL-22, secreted by NK cells, also has this effect on tumor cells (Supporting Information Figure 4). Future studies addressing this question are of significance to elucidate the function of these NKp44⁺ NK cells in cancer.

As demonstrated by us and others, PGE2 induces expression of CCR7 on DCs and functionally increases their migratory responsiveness to lymph node-homing chemokines *in vitro* (22, 35, 39). Based on these observations, PGE2 is often incorporated in DC maturation cocktails used in clinical vaccination studies. Our data on the inhibitory effect of PGE2 on NK-DC interaction in combination with previous reports on enhanced T_H17 and T_H2 induction by PGE2 matured DCs and the decreased ability to induce antigen specific CTLs (35) argue against the use of PGE2-matured DCs in dendritic cell-based vaccines, as it does not contribute to *in vivo* DC-migration and has detrimental effects on immune effector mechanisms. In addition, Muthuswamy et al. showed that increased CCR7 expression of PGE2 matured DCs, as compared to TLR-matured DCs, correlates with their lack of CCL19 production (48). If TLR-matured DCs, that express low levels of CCR7, are cultured in an environment with low concentrations of CCL19, CCR7 expression is increased. Additionally, they showed in a clinical trial that PGE2-matured DCs demonstrated no advantage over TLR-triggered DCs in lymph node homing. In the current study, we show that the effect of PGE2 is imprinted during IFN- γ /FMKp DC maturation since cytokine and chemokine secretion is only affected by PGE2 in the first 6 h of maturation and this effect is mainly mediated via EP2 and EP4 and depends on cAMP signaling. In terms of vaccination protocols, these data support the application of *ex vivo* matured DCs in patients with tumors secreting PGE2. Moreover, our data indicate that PGE2 does not induce permanent changes in NK cell cytotoxic capacity, since the suppressive effect of PGE2 on NK cells can be easily overcome by DC-induced NK cell activation. However, to effectively activate NK cells, NK-DC interaction should take place in an environment with low concentrations of PGE2, emphasizing the need of NK cell

recruitment by DCs. Our results also open the possibility to combine DC vaccination with COX2-inhibitory therapy, which has previously been shown in mouse models to enhance the efficacy of cancer vaccines (49, 50).

In conclusion, we report on a novel immunosuppressive effect of PGE2, mediated by IFN- γ /FMKp matured DCs, on NK cell function. These current data help to understand the complex role of PGE2 in the regulation of immune responses during inflammation. The extent of effect of PGE2 on the immune system in relation to other tumor-derived factors and thereby the redundancy of this mechanism is yet not completely elucidated and *in vivo* studies should be performed to answer this question. However, we show *in vitro* that the immunosuppressive effect of PGE2 on NK-DC interaction possibly represents one of many tumor-mediated mechanism to hamper acute immune responses.

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Supporting information

Figure. 1. Effect of PGE2 on surface expression of maturation markers by DCs matured with IFN γ /FMKp and migration toward CCL19.

Monocyte-derived DCs were differentiated for 5 days by IL-4 and GM-CSF to immature DCs. Maturation was induced by IFN- γ /FMKp in the presence or absence of 5 μ g/ml PGE2. After 6 h maturation, DCs were washed to remove the stimulation medium, DCs were matured for an additional 18 h in AIM-V[®] medium only. (A) Surface expression of HLA-DR, CCR7, CD80, CD83, CD86 and CD40 was evaluated by flow cytometric analysis. Gray filled histograms represent immature DCs, solid black lines-IFN γ /FMKp matured DCs and red line represent IFN- γ /FMKp matured DCs in the presence of 5 μ g/ml PGE2. Representative data of one out of four different donors. (B) Expression of CCR7 as evaluated by flow cytometry. Results are presented as mean MFI plus SEM and are obtained from 3 different donors. (C) Percentage of DCs specifically migrated toward a CCL19 gradient (250 ng/ml). Results are presented as mean plus SEM and are obtained from 3 different donors that were analyzed in triplicate. ANOVA significance * $P < 0.05$, ** $P < 0.01$.

Figure 2. Phenotype, cytokine production and NK cell activating properties of blood-derived BDCA1+ DCs.

BDCA1+ DCs were isolated from 500 ml of fresh blood using an immunomagnetic, negative isolation kit (Miltenyi Biotec). Cells were seeded at a density of 5×10^5 cells/ml in a 24-well plate and matured with IFN- γ /FMKp or with IFN- γ /FMKp in the presence of PGE2. 6 h after maturation induction, the maturation stimuli were removed by extensive washing and DCs were incubated in serum-free medium for 42 h. (A) Flow cytometric characterization of differently matured BDCA1+ DCs. Gray, filled histograms represent the surface expression observed in non-matured BDCA1+ DCs, black line histograms represent the IFN- γ /FMKp matured and red line histograms represent IFN- γ /FMKp BDCA1+ DCs matured in the presence of PGE2. Representative data of 2 independent experiments are shown. (B) CCL5 production by differently matured BDCA1+ DCs as measured by ELISA. Data show the mean + SD of 2 independent experiments. (C) IFN- γ secretion of NK cells cultured in the supernatant of the three differently matured BDCA1+ DCs. Fresh NK cells were cultured in the supernatant of differently matured BDCA1+ DCs. After 24 h the supernatant was harvested and IFN- γ was quantified with ELISA. NK cells from 2 different donors were cultured in supernatants of 2 different BDCA1+ DC donors. (ANOVA significance $*P < 0.05$).

Figure 3. Effect of A549 cell-derived factors on NK-DC interaction. (A) IL-12 secretion of IFN-γ/FMKp matured DCs in serum-free or A549 tumor cell-conditioned medium with or without PGE2. The A549 cell line was cultured for 48 h in serum-free AIM-V[®] medium in presence or absence of (250μM) arachidonic acid (AA). Immature DCs were matured either in tumor cell-conditioned medium (PGE2-producing or not) or in AIM-V[®] supplemented with IFN-γ/FMKp in the presence or absence of Rh-PGE2 (recombinant). After 6 h, maturation stimuli were washed away and DCs were incubated for an additional 18 h in AIM-V[®]. Supernatant was collected and IL-12 was determined by ELISA. Data show the mean plus SEM of 2 donors. (B) NK cell-derived IFN-γ production in supernatant of IFN-γ/FMKp DCs matured in AIM-V[®] or tumor cell-conditioned medium in presence or absence of PGE2 (recombinant or tumor cell-derived). Freshly isolated NK cells were co-cultured for 16 h with differently matured DCs. IFN-γ was quantified using Cytometric Bead Array. Shown results represent the mean plus SEM of NK cells from 2 different donors added to the supernatants of 2 different DC donors (n=4). (C) The A549 cell line was cultured for 4 days in serum free medium with (250μM) or without arachidonic acid. TGF-β and PGE2 were quantified using ELISA.

Figure 4. Model of the inflammation restraining effects of PGE2 on NK-DC interaction on tumor development.

Presence of tumor-derived PGE2 during DC maturation inhibits the production of NK cell-recruiting chemokines (CCL5, CCL19, CXCL10) and thereby decreases NK cell recruitment. Additionally, PGE2 reduces IL-18 production by DCs and shifts the IL-12/IL-23 balance toward IL-23 production. This results in decreased IFN- γ production and cytotoxic capacity of peripheral blood NK cells and enhanced secretion of IL-22 by NK cells residing in mucosa-associated lymphoid tissue. Decreased IFN- γ production of NK cells together with the altered cytokine profile of PGE2 matured DCs is responsible for inhibition of T_H1 polarization. Tumor development is possibly stimulated by three independent effects of PGE2: 1) Direct effect of PGE2 on tumor cell proliferation and apoptosis, 2) decreased killing of tumor cells by NK cells that were either directly stimulated with PGE2 or interacted with PGE2 triggered DCs and 3) effects of IL-22, produced by NK cells that interacted with PGE2 stimulated DCs, on tumor cell proliferation and apoptosis.

Chapter

6

Flow Cytometry-based Assay to Evaluate Human Serum MUC1- Tn Antibodies

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Abstract

Mucin-1 (MUC1) is a heavily O-glycosylated, transmembrane protein that is expressed on the apical surface of most secretory epithelia. In malignantly transformed epithelia, MUC1 has lost its apical distribution, is underglycosylated and is secreted into the circulation. Due to the underglycosylation of MUC1, cancer-specific MUC1-Tn/STn antigens, which are highly immunogenic, become exposed. We aimed at developing a system that allows detection of antibodies directed to the native form of MUC1 and the underglycosylated MUC1-Tn epitopes. To this end, we made use of the Chinese Hamster Ovary (CHO) *Id1D* cell line stably transfected with MUC1. This cell line has a glycosylation defect, which can be reversed by addition of different monosaccharides to the cell culture and enables the production of cells expressing the MUC1-Tn glycoforms. After validation with glyco-specific antibodies, the CHO-*Id1D* MUC1 system was used to detect serum MUC1 and MUC1-Tn antibodies. Using this system, we could confirm the presence of MUC1-Tn antibodies in the serum of a patient vaccinated with a truncated MUC1 peptide. This indicates that the CHO-*Id1D* MUC1 system represents a flow cytometry-based technique to detect antibodies binding to the underglycosylated MUC1 protein. This cellular system is complementary to previously published methods to detect MUC1 serum antibodies, since the antibodies to the native protein are evaluated and therefore it can be effectively used for MUC1 antibody monitoring in vaccination studies as well as for functional assays.

Introduction

The transmembrane protein Mucin-1 (MUC1) is a heavily glycosylated protein, which is expressed on the apical surface of most secretory epithelia as well as on a variety of hematopoietic cells (1, 2). The extracellular domain of MUC1 consists of a variable number of 20 amino acid tandem repeats (HGVTSAPDTRPAPGSTAPPA). Within each tandem repeat, two serines and three threonines represent five potential *O*-glycosylation sites that are extensively glycosylated (Figure 1). The extent of glycosylation depends on the expression of tissue-specific glycosyltransferases (2).

In most adenocarcinomas and some hematological malignancies, it has been demonstrated that MUC1 is overexpressed, lost its apical distribution and is secreted into the circulation (3-7). Moreover, the extracellular MUC1 domain is aberrantly glycosylated, which is caused by upregulation of sialyltransferases and downregulation of glycosyltransferases resulting in premature termination of glycosylation (8, 9). Altered MUC1 expression has been shown to increase tumorigenicity, by at least four different mechanisms. First, altered MUC1 expression has been coupled with enhanced metastasis formation due to direct binding of cancer-associated MUC1 to ligands augmenting cancer cell-endothelial cell adhesion (10). Second, signaling of the intracellular MUC1 domain is responsible for stabilization of growth factor receptors thereby enhancing cell proliferation (11). Third, MUC1 directly binds p53 inducing decreased production of apoptotic genes thereby supporting cell survival (12). Fourth, overexpression of MUC1 can reduce intercellular adhesion due to steric hindrance, allowing tumor cells to escape from immune recognition (13).

Next to the tumor supporting capacity of MUC1, alteration of MUC1 can also increase the immunogenicity of tumor cells. Due to decreased MUC1 glycosylation, new tumor-associated epitopes, which were normally masked by large sugar moieties, become exposed (14). MUC1-associated antigens frequently expressed in cancer are the immunogenic Tn (GalNAc-) and T (Gal β 1-3GalNAc-) antigens along with their sialylated versions (ST and STn) (15, 16). Since it has been suggested that these antigens are not subjected to immune-tolerance, they represent attractive tumor associated antigens to target with immunotherapy (17, 18).

Figure 1. Schematic presentation of O-glycosylation of MUC1.

O-linked glycosylation is initiated by (a) polypeptide N-acetylgalactosaminyltransferases (GalNAc-Ts), which links N-acetylgalactosamine (GalNAc) to a serine (Ser) or threonine (Thr) of the polypeptide backbone and results in the formation of the Tn antigen. This can be followed by sialic acid linkage to the GalNAc moiety, which is catalyzed by (b) sialyltransferase ST6GalNAc-I, and as a consequence the sialyl-Tn (STn) antigen is formed. If not sialylated, elongation of glycosylation can either form core 3 and 4 antigens or core 1 structures may be synthesized by (c) galactosyltransferase β 3GalT, which add galactose (Gal) to the GalNAc moiety, resulting in the T (core 1) antigen. Also the T antigen can become sialylated creating ST antigens by either linking sialic acid to the GalNAc moiety, catalyzed by (d) ST6GalNAc-I, II or to the Gal moiety, catalyzed by (e) ST3Gal-I. Additionally both Gal and GalNAc can become sialylated. Linkage of more sugar moieties to the Tn antigen will result in the elongation of Core 1 structures or formation of core 2 antigens.

The immunogenicity of MUC1 has been confirmed by the detection of anti-MUC1 antibodies in the serum of healthy controls, as well as cancer patients (19). In many malignancies it has been demonstrated that MUC1 serum antibodies, which are able to

mediate antibody-dependent cellular cytotoxicity (ADCC) (20), are increased and correlate with a better prognosis (4, 21). However, in some cancer patients the presence of MUC1 antibodies is decreased, due to immune complex formation with circulating MUC1 (4, 22). It can be anticipated that enhancement of anti-MUC1 immune responses by immunotherapy could induce strong antitumor responses. Studies using MUC1 antibody-, MUC1 peptide-, or DC-based vaccination strategies have been shown to be safe and feasible (17, 23-25). Additionally, these studies show promising results by boosting the immune response of cancer patients.

Besides mounting clinical outcome, there is an urgent need for tools which monitor immune responses against tumor-associated, aberrant MUC1 glycosylation patterns. It can be anticipated, that monitoring of humoral immune responses with a system that detects responses to underglycosylated MUC1 might correlate better with clinical responses than evaluation with the current available techniques (26). Therefore, it is relevant to develop a system in which MUC1 glycosylation can be manipulated to detect immune responses against these underglycosylated MUC1 epitopes.

In this study, we present a flow cytometry-based method to evaluate the presence of antibodies directed to MUC1-Tn epitopes in serum. To this end, we made use of the Chinese Hamster Ovary (CHO) *IdID* cell line, which is deficient in its UDP-galactose/UDP-N-acetylgalactosamine 4-epimerase enzyme (27, 28). This enzyme is crucial for the formation of UDP-Gal and UDP-GalNAc from UDP-Glc/GlcNAc and as a consequence both N-linked and O-linked glycosylation are affected by the defect. The glycosylation can be restored by providing the CHO-*IdID* cell with exogenous sources of Gal and GalNAc (27).

We used the CHO-*IdID* cells stably transfected with a full coding sequence of the MUC1 protein (32 tandem repeats), enabling the production of cells expressing specific MUC1 glycoforms. After validation of this system by glycosylation-specific as well as MUC1-specific antibodies, we used these cells to screen antibodies recognizing MUC1-Tn epitopes in sera from breast cancer patients, healthy controls and a breast cancer patient vaccinated with a keyhole limpet hemocyanin-conjugated truncated MUC1 peptide.

Materials and methods

Cell culture

CHO-*IdlD* and CHO-*IdlD* cells stably transfected with MUC1F were cultured in Iscove's Modified Dulbecco Medium supplemented with 3% FBS, 1% penicilline/streptomycin and 600 µg/ml G418. The UDP-Gal/UDP-GalNAc 4-epimerase deficient CHO-*IdlD* MUC1 cells and the CHO-*IdlD* cells, which served as a negative control, were cultured for 3 days with 1mM GalNAc (Sigma-Aldrich, St. Louis, MO, USA), inducing them to express Tn-MUC1 or with 1mM GalNAc and 0.1 mM Gal (Sigma-Aldrich), inducing them to express T-MUC1 (Figure 1).

Serum

Frozen serum (-20°C) of five healthy controls and seven breast cancer patients were obtained from the department of clinical chemistry (Maastricht University Medical Center[†]). A positive serum sample, from a breast cancer patient, vaccinated with a keyhole limpet hemocyanin-conjugated truncated MUC1 peptide, expressing anti-Tn-MUC1 antibodies was used as a positive control (29, 30).

Antibodies

MUC1 antibody 214D4 (purified from the supernatant of the 214D4 cell line (31)) was kindly provided by Dr. J. Hilkens (the Netherlands Cancer Institute, Amsterdam, the Netherlands), MAb 5E5 (16) and MAb 5F4 (32) were used for flow cytometric evaluation of MUC1 epitope expression by CHO-*IdlD* MUC1 cells. A detailed description of the specificities of the MUC1 antibodies used in this study has been published previously (6). Briefly, the MAb 214D4 recognizes human MUC1 irrespective of its glycosylation pattern, MAb 5E5 exclusively recognizes MUC1-Tn/STn and MAb 5F4 recognizes Tn epitopes irrespective of peptide backbone they are associated with.

Flow cytometry

CHO-*IdlD* and CHO-*IdlD* MUC1F cells supplemented with either Gal, GalNAc, or Gal and GalNAc were incubated with different antibodies (MAb 214D4, 5E5 or 5F4), washed and

incubated with the secondary antibody goat-anti-mouse R-phycoerithrin (PE) labelled (BD Biosciences, San Jose, CA, USA). For the detection of human serum antibodies, CHO-*IdlD* and CHO-*IdlD* MUC1 cells supplemented with either Gal, GalNAc, or Gal and GalNAc were preincubated with rat serum, washed and incubated with 20 and 40 times diluted serum. Cells were then incubated with a secondary Donkey-anti-Human R-phycoerithrin (PE) labelled antibody, which is preabsorbed for rat (Jackson ImmunoResearch, West Grove, PA, USA). 7-amino-actinomycin D (7AAD) was used to differentiate between viable and dead cells. All antibody incubations were performed at 4°C for 1h. Reactivity of antibodies with the CHO-*IdlD* and CHO-*IdlD* MUC1F cells was analysed by flow cytometry using a BD FACSSort (BD Biosciences) and data were analyzed with BD CellQuest™ Pro Software (BD Biosciences).

Results

Detection of O-glycosylation of MUC1 after GalNAc and/or Gal supplementation

To confirm surface expression of MUC1 by the CHO-*IdlD* MUC1 cells, reactivity of the cells with MAb 214D4, recognizing MUC1 irrespective of its glycosylation was analysed. The results of flow cytometric analysis showed that the non-transfected CHO-*IdlD* cells do not bind with the 214D4 antibody, whereas the CHO-*IdlD* MUC1 cells do (MFI of 4,43 and 210, respectively) (Figure 2A).

To evaluate whether the glycosylation defect of the CHO-*IdlD* cells can be reversed by supplementing the culture medium with GalNAc and/or Gal, we performed binding experiments with antibodies specific for different MUC1-associated, O-glycan structures (or O-glycan haptens). O-glycosylation of MUC1 is initiated after binding of GalNAc to one of the glycosylation sites (threonine or serine), creating the MUC1-Tn epitope. Thereafter, glycosylation is continued by linking of Gal to the first GalNAc (Figure 1). To induce glycosylation, CHO-*IdlD* and CHO-*IdlD* MUC1 cells were cultured for 3 days in the presence of GalNAc, Gal or a combination of both GalNAc and Gal. The cells were then harvested and binding with MAb 5E5, which specifically recognizes the combined glycopeptide epitope MUC1-Tn/STn (16), was assessed with flow cytometry. When CHO-*IdlD* MUC1 cells

were cultured in medium supplemented with GalNAc, a shift in 5E5 binding signal was observed as compared to CHO-*Id1D* MUC1 cells cultured without sugar (MFI increased from 25 to 213) (Figure 2B).

This shift in 5E5 binding signal was not observed in untransfected CHO-*Id1D* cells incubated with GalNAc. In addition to 5E5 MAb binding, MUC1 glycosylation was further analyzed by staining with MAb 5F4, which recognizes Tn epitopes irrespective of the peptide backbone. Also with this antibody, a shift in binding signal was observed when CHO-*Id1D* MUC1 cells were cultured in GalNAc-containing medium (MFI increased from 6,8 to 33) (Figure 2B). These shifts in 5E5 and 5F4 binding indicate that supplementation with GalNAc results in MUC1-Tn epitope formation.

When in addition to GalNAc also Gal was added to the medium, decreased binding of MAb 5E5 was detected (Figure 2B), indicating that glycosylation proceeds after Gal linking to the first GalNAc. In contrast, neither supplementation of Gal alone to the CHO-*Id1D* MUC1 cells nor the addition of any monosaccharide to the CHO-*Id1D* cells resulted in the formation of MUC1-Tn epitopes (data not shown). These data reveal that it is possible to reconstitute MUC1 O-glycosylation in the CHO-*Id1D* MUC1 cells by GalNAc supplementation, which results in MUC1-Tn epitope formation and if Gal is also supplemented the surface expression of these epitopes decreases (MFI 6.9).

Figure 2. Expression of MUC1 and MUC1-Tn epitopes by CHO-*Id1D* MUC1 cells as analyzed by flow cytometry.

CHO-*Id1D* and CHO-*Id1D* MUC1 cells were cultured for 3 days in the presence or absence of GalNAc, Gal or the combination of GalNAc and Gal. Analysed cells were gated on 7AAD⁻. (A) Binding of MAb 214D4 (recognizing MUC1) by CHO-*Id1D* cells and CHO-*Id1D* cells transfected with MUC1.

Figure 2. Expression of MUC1 and MUC1-Tn epitopes by CHO-IdID MUC1 cells as analyzed by flow cytometry (continued). (B) Binding of MAb 214D4, 5E5 (recognizing MUC1-Tn/STn) or MAb 5F4 (recognizing STn) by CHO-IdID and CHO-IdID MUC1 cells cultured with or without GalNAc or GalNAc and Gal. ND, not determined.

Optimization of CHO-IdID MUC1 flow cytometric analysis

The use of CHO-IdID cells for flow cytometric analysis is complicated by the culture characteristics of the CHO-IdID cells and therefore needed optimization. Since the CHO-IdID cells scavenge the medium for free Gal and GalNAc, they must be cultured at low serum concentrations, to preserve the glycosylation defect. Additionally, because CHO-IdID cells are adherent, the generation of a single cell suspension is accompanied by cell death. Dead cells are responsible for aspecific binding of antibodies and co-staining with 7AAD showed that in the 7AAD positive population there is a subpopulation clearly

positive for the MUC1 antibodies (Figure 3A). Moreover, reactivity with isotype antibody control could be detected, confirming the aspecific staining of the 7AAD positive cells.

To decrease the amount of dead cells and increase the yield, two different harvesting techniques were evaluated. Cell scraping was compared with trypsinization of the CHO-*IdID* MUC1 cells. In contrast to cell scraping, trypsinization reduced the number of dead cells by 30%, however flow cytometric analysis showed that trypsinization induced expression of new epitopes reactive with the MUC1 specific antibodies, but not isotype control antibody (Figure 3B). Cleaving of the MUC1 peptide by trypsin could be responsible for this new epitope formation. Even though cell scraping induces a lot of cell death, it remains the preferred option in this system since death cells can be excluded using 7AAD staining.

Figure 3. Optimization of CHO-*IdID* culture for flow cytometric analysis.

(A) CHO-*IdID* cells were cultured in monolayer were scraped, incubated with isotype (IgG1) and anti-MUC1 antibodies (MAb 214D4 and 5E5). Before analysis cells were stained with 7AAD. Without gating (left panel) a population of CHO-*IdID* cells is positive for all antibodies, which is due to the two subpopulations of 7AAD⁺ cells (middle panel) and which not present after gating on 7AAD⁻ cells (right panel). (B) Comparison antibody (isotype, 214D4, 5E5) binding of CHO-*IdID* MUC1 cells after being scraped or trypsinized.

Detection of serum antibodies directed to MUC1-Tn/STn epitopes in healthy controls and (vaccinated) breast cancer patients.

As shown in figure 2, the CHO-*ldID* MUC1 system is effective in generating MUC1-Tn epitopes. To analyse if MUC1-Tn antibodies are present in sera of breast cancer patients as well as in healthy controls, CHO-*ldID* MUC1 cells were cultured in the presence or absence of GalNAc and prior to flow cytometric analysis cells were incubated with human serum. The CHO-*ldID* cells were taken along as a negative control, to exclude aspecific or specific reactivity. Secondary antibody staining was performed for detection of serum antibodies to MUC1 and Tn-MUC1. Both anti-human IgM- and IgG-detecting secondary antibodies were used to discriminate between primary (IgM) and secondary humoral responses (IgG). Healthy controls as well as breast cancer patients did not show specific binding of serum antibodies with CHO-*ldID* MUC1 cells cultured with or without GalNAc. Even though in the serum of breast cancer patients repetitively a marginal shift of the histogram could be observed when a secondary IgM-recognizing antibody was used (Figure 4A).

In contrast, a post-vaccination serum sample of a breast cancer patient vaccinated with a keyhole limpet hemocyanin-conjugated truncated MUC1 peptide, which was tested positive for antibodies directed to aberrantly glycosylated MUC1 by O-glycopeptide microarray (33), showed an increased binding signal after incubation with CHO-*ldID* MUC1 cells (Increase in MFI of anti-IgG binding from 53.7 to 127 and of anti-IgM binding from 5.4 to 9.4). Moreover, both IgG and IgM antibodies directed to MUC1-Tn epitopes were present in this serum (increase in MFI of anti-IgG binding from 91.7 to 143 and of anti-IgM binding from 8.4 to 12.9). To confirm that reactivity to CHO-*ldID* MUC1 cells cultured with GalNAc was actually directed to MUC1 Tn epitopes and not to the MUC1 protein, antibody reactivity to CHO-*ldID* MUC1 cells cultured with GalNAc and Gal, restoring glycosylation, was analyzed. No antibody reactivity was detected if serum was incubated with these CHO-*ldID* MUC1 cells (Figure 4B), indicating that the antibodies were indeed MUC1 Tn specific.

Figure 4. Presence of MUC1-Tn antibodies in serum of breast cancer patients and healthy controls.

CHO-*IdlD* and CHO-*IdlD* MUC1 cells were cultured for 3 days in the presence or absence of GalNAc, Gal or the combination of GalNAc and Gal. Cells were incubated with serum, analyzed by flow cytometry and gated on 7AAD⁻. (A) Expression of MUC1 and MUC1-Tn IgG and IgM antibodies by breast cancer patients (representative data of one out of seven patients), healthy controls (representative data of one out of five patients) or a breast cancer patient, vaccinated with a keyhole limpet hemocyanin-conjugated truncated MUC1 peptide (this sample was tested in two independent experiments). Only the serum from the vaccinated patient contained MUC1-Tn IgG and IgM antibodies and thus reacted with the CHO-*IdlD* MUC1 cells cultured with GalNAc, however (B) when CHO-*IdlD* MUC1 cell were cultured in the presence of both Gal and GalNAc, reactivity with the anti-IgG antibody decreased.

Discussion

In the present study we describe a flow cytometric method to detect both MUC1 and MUC1-Tn antibodies in human serum. To this end, we used CHO-*IdlD* cells stably transfected with MUC1. Due to its UDP-Gal/UDP-GalNAc 4-epimerase enzyme deficiency,

the glycosylation of MUC1 can be effectively manipulated by addition of different monosaccharides. Supplementation of GalNAc to the cell culture results in the formation of the cancer-associated MUC1-Tn epitope that can be detected by flow cytometry using glycospecific MUC1 antibodies. Additionally, the detection of these MUC1-Tn epitopes is decreased after supplementation of both Gal and GalNAc, which presumably is caused by extension of glycosylation. The capacity of CHO-*IdID* cells to express MUC1-Tn antigens, as detected by cytospin analysis, has been reported by Sørensen et al. (17). In this report, we extend these observations by showing that expression of MUC1 and MUC1-Tn epitopes can also be detected with flow cytometry, which is a more sensitive method that quantifies expression (34).

With the CHO-*IdID* MUC1-based flow cytometric assay, we do not detect serum antibodies against the unglycosylated MUC1 protein in non-vaccinated breast cancer patients. However, both IgG and IgM antibodies can be detected in the serum of a breast cancer patient vaccinated with a truncated MUC1 peptide, indicating that immune responses induced by immunotherapy can be detected with this flow cytometric system. Detection of antibodies against unglycosylated MUC1 seems to be in apparent contrast with previous reports by Altschuler et al, who showed that CHO-*IdID* cells rapidly endocytose and degrade non-glycosylated surface MUC1 (35). Nevertheless, MUC1 expression can be detected by flow cytometry with MAb 214D4 when the CHO-*IdID* culture is not supplemented with any carbohydrate, indicating that CHO-*IdID* still express non-glycosylated surface MUC1.

Detection by ELISA and Western Blot of anti-MUC1 antibodies in serum of cancer patients as well as healthy controls has been described previously. However, the percentage of patients and controls expressing these antibodies show large variations between studies (4, 36-39). In some studies, MUC1 serum antibodies could not be detected in healthy controls (39), whereas other studies demonstrated that up to 16% of healthy controls show reactivity to MUC1 peptides (37). In cancer patients, the reported levels of anti-MUC1 antibodies also differ, due to the presence of soluble serum MUC1. Depending on tumor type, these serum MUC1 antigens have been shown to complex with anti-MUC1 antibodies (4). Standardization of the different methods, including the flow cytometric

assay we describe, seems to be necessary to answer the question on prevalence of anti-MUC serum antibodies in healthy controls and cancer patients. The number of samples tested in this study do not justify a conclusion on prevalence of these antibodies, we merely show that with this technique we are able to detect human serum antibodies directed to MUC1 and underglycosylated MUC1.

In addition to the detection of serum antibodies against unglycosylated MUC1, manipulation of MUC1 glycosylation in the CHO-*Id1D* MUC1 system allowed us to selectively test for the presence of IgG and IgM antibody responses to MUC1-Tn. These serum antibodies could only be detected in a breast cancer patient after vaccination and not in non-vaccinated cancer patients or healthy controls. Detection of antibodies directed to underglycosylated MUC1 has been recently described by Wandall et al. (29), who made use of an O-glycopeptide microarray to demonstrate that MUC1-Tn/STn associated IgG serum antibodies are present in low numbers of newly diagnosed breast, ovarian and prostate cancer patients and not in healthy controls. Additionally, in patients who had no pre-existing MUC1-Tn/STn IgG antibodies, it was shown that they did develop detectable serum IgG and IgM MUC1-Tn antibodies after vaccination. Similar findings were previously described by Sabbatini et al. (30), who demonstrated that MUC1-Tn antibodies could be detected by ELISA.

Both, ELISA and O-glycopeptide microarrays make use of small MUC1 peptides that are differently glycosylated. The O-glycopeptide microarray allows rapid mapping of serum antibody specificity and has already been proven to be reliable in detection of MUC1 serum antibodies in mice vaccination studies (40). Even though the glycosylation sites can be controlled in the small peptide-based methods, allowing specific antibody mapping, these methods are only able to detect antibodies binding to linear MUC1 structures. In contrast to this highly sensitive method, which can be efficiently used for detection of cancer biomarkers, the CHO-*Id1D* MUC1 system is able to detect antibodies to the native, three dimensional MUC1. Moreover, since it is a cellular system, it is possible to do *in vitro* functionality studies like ADCC and complement activation. This makes the CHO-*Id1D* MUC1 system complementary to the previously published methods to detect MUC1

serum antibodies, since the clinical significance of underglycosylated MUC1-antibodies in relation to cancer is largely unknown.

In conclusion, we report on a cellular, flow cytometry-based technique to detect serum MUC1-Tn antibodies. We show that it is a unique system to detect antibodies binding to the native underglycosylated MUC1 protein and can be effectively used for antibody monitoring and functional assays.

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Chapter

7

In vitro differentiated T/NK cell progenitors derived from human CD34⁺ cells mature in the thymus

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Abstract

Haploidentical hematopoietic stem cell transplantation (haplo-HSCT) is a treatment option for patients with hematopoietic malignancies that is hampered by treatment-related morbidity and mortality, in part the result of opportunistic infections, a direct consequence of delayed T cell recovery. Thymic output can be improved by facilitation of thymic immigration, known to require precommitment of CD34⁺ cells. We demonstrate that Deltalike ligand-mediated predifferentiation of mobilized CD34⁺ cells in vitro results in a population of thymocyte-like cells arrested at a T/natural killer (NK) cell progenitor stage. On intrahepatic transfer to Rag2^{-/-}γc^{-/-} mice, these cells selectively home to the thymus and differentiate toward surface T cell receptor-αβ⁺ mature T cells considerably faster than animals transplanted with noncultured CD34⁺ cells. This finding creates the opportunity to develop an early T-cell reconstitution therapy to combine with HSCT.

Introduction

Haploidentical hematopoietic stem cell transplantation (haplo- HSCT) has shown impressive results in the treatment of leukemia (1, 2). HSCT is hampered by treatment-related mortality resulting from opportunistic infections (2-4). One obvious causative factor for enhanced susceptibility to pathogens is the slow recovery of T cells in the periphery (3, 5). In mice, it is established that T-lineage-directed progenitors generated in vitro have the capacity to migrate to the thymus and complete their development into host-tolerant T cells (6, 7). Because repopulation of the thymus after transplantation is considered a rate-limiting process, supplementation of in vitro pre-T-lineage-directed cells may be one approach with potential to improve T cell levels after HSCT. However, not much is known about the in vitro T-lineage commitment potential of granulocyte colony-stimulating factor mobilized CD34⁺ (mCD34⁺) cells commonly used for haplo-HSCT. To stimulate development of T-lineage-directed cells, we use the TSt-4 murine thymic stromal cell line (8) transduced with human Delta-like ligand (hDLL), demonstrated to have the capacity to generate T-lineage-directed cells from cord blood (CB) CD34⁺ cells (9). Importantly, the T-lineage-directed cells were arrested at the CD5⁺CD7⁺ stage, considered to be the murine equivalent of double-negative 2 or 3 thymocytes, the designated stage of T-lineage development suitable for direct thymic reconstitution. The goal of this study is to examine the potential of mCD34⁺ cells to generate T-lineage-directed progenitors, determine whether this potential is influenced by a specific Notch ligand, and investigate whether mCD34⁺ cell-derived progenitors have the capacity to home to and mature in the thymus.

Materials and methods

Cells

Mobilized CD34⁺ stem cells were isolated from peripheral blood mononuclear cells (PBMC) obtained from healthy volunteers (V1–3) treated with G-CSF (Neupogen, Amgen Inc., Thousand Oaks, CA) at MUMC⁺ (Maastricht, The Netherlands). Isolation was performed with the Isolex 300i Magnetic cell selection system v2.5 (Baxter oncology, Brussels, Belgium) using the Isolex stem cell reagent kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The positive fraction was frozen at a concentration of 5×10^6 cells per ml per vial. Purity of mobilized and CB cells, obtained after informed consent, was more than or equal to 95% CD34⁺ cells and less than or equal to 0.1% contaminating CD3/CD56⁺ cells. Informed consent was obtained from all CD34⁺ cell donors in accordance with the Declaration of Helsinki. TSt-4 cell lines (8-10) and K562 were grown in standard media.

Fresh human CB was obtained with parent written informed consent from healthy full-term newborns (Department of Gynecology and Obstetrics, Ospedale San Giovanni, Bellinzona, Switzerland). After density gradient centrifugation, CD34⁺ CB cells were enriched using immunomagnetic beads according to the manufacturer's instructions (CD34⁺ selection kit, Miltenyi). Samples were evaluated by FACS for CD34⁺ and CD3⁺ cell content. If CD34⁺ purity was < 95% and/or CD3⁺ cells were $\geq 0.1\%$, the sample was enriched again until these values were met, after which cells were frozen.

Natural killer (NK) cells were enriched from peripheral blood of healthy donors using the NK isolation kit according to the manufacturer's instructions (Miltenyi Biotec).

Thymic stromal cell lines TSt-4 (8) and TSt-4 transduced with hDLL1 or hDLL4 (10) were maintained in RPMI 1640 (Sigma-Aldrich Co., St. Louis, MO) containing 5% fetal bovine serum (FBS, Greiner Bio-one, Solingen, Germany), 1% penicilline-streptomycine (PS), 1 mM sodium pyruvate, 0.1 mM MEM non essential amino acids (NEAA), and 5×10^{-5} M 2-mercaptoethanol (β ME) (all from Invitrogen Ltd., Paisley, UK). The HLA Class I negative erythro-myeloid leukemia cell line K562 was grown in RPMI 1640 (Invitrogen) supplemented with 10% FBS and 1% PS.

Flow cytometry

All antibodies, materials and equipment were obtained from BD Biosciences (Erembodegem, Belgium), unless stated otherwise. All materials were used according to the manufacturer's instructions. At different time points, cells were analyzed using different combinations of fluorescein isothiocyanate (FITC), R-phycoerithrin (PE), PE-Cyanine dye 7 (PE-Cy7), peridinin chlorophyll-a protein (PerCP)-, allophycocyanin (APC), and APC-H7 conjugated monoclonal antibodies (mAbs). The following antibodies (clones) were used: CD1a (HI149), CD2 (6G4- Sanguin, Amsterdam, The Netherlands), CD3 (UCHT1, SK7), CD4 (SK3, 13B8.2 – Beckman Coulter, Fullerton, CA), CD5 (UCHT2, L17F12, MEM32 – Immunotools, Friesoythe, Germany), CD7 (M-T701, 7F3- Sanquin, 124-1D1- eBioscience (San Diego, CA), 6B7- Caltag, Burlingame, CA), CD8a (HIT8a, RPA T8), CD14 (M5E2), CD19 (HIB19), CD34 (8G12), CD38 (HB7), CD45 (2D1, HI30), CD45RA (HI100, MEM-56- Caltag), CD56 (B159, N901- Beckman Coulter), CD107a (H4A3), CD158a (HP-3E4), CD158b (CH-L), IFN- γ (25723.11), NKG2A (131411, R&D Systems, Minneapolis, MN), NKp46 (9E2), TCR $\alpha\beta$ (IP26- eBioscience), TCR $\gamma\delta$ (B1.1- eBioscience). 7-amino-actinomycin D (7AAD), 4',6-diamidino-2-phenylindole (DAPI), or propidium iodide (PI) was used to differentiate between viable and dead cells. For intracellular stainings, cells were permeabilized using perm/wash. Cells were analyzed on a FACSCalibur, FACScan, or FACSCanto II, with FACSDiva (BD), WinMDI (Joe Trotter, <http://facs.scripps.edu/>) or FlowJo (Treestar, Ashland, OR) software.

Prior to differentiation, CD34⁺ cell preparations were depleted of CD38⁺ cells and contaminating T and NK cells by cell-sorting with a FACSARIA using conjugated anti-CD3, -CD4, -CD8, -CD38 and -CD56 antibodies. All mCD34⁺ cell preparations had < 0.05% contaminating mature lymphoid cells.

Co-culture of mCD34+ cells with stromal cells

mCD34⁺ cells were seeded at a density of 2.6×10^3 cells/cm² on monolayers of TSt-4 cells expressing Notch ligands DLL1 or DLL4. During the 1st week, cultures were maintained in RPMI 1640 containing 10% FBS, 1% PS, sodium pyruvate, NEAA, and bME that was supplemented with 100 ng/ml Stem Cell Factor (SCF), 100 ng/ml Fms-related tyrosine kinase 3 Ligand (Flt3-L), 50 ng/ml Thrombopoietin (TPO) (all from PeproTech, New York,

NY) and 10ng/ml Interleukin 7 (IL-7, R&D Systems). After 1 week, co-cultures were maintained in complete medium supplemented with IL-7 (20 ng/ml). Co-cultures were refreshed by halve medium change 3 times weekly. Differentiating cells were transferred to fresh monolayers every 2 weeks. For transfer, cells were separated from the monolayers by disruption of the monolayers using cell scrapers (BD), resuspension by pipetting, and filtration through a 100 µm mesh (Nedfilter, Almere, The Netherlands) directly onto the new monolayer. IL-15 (20 ng/ml, R&D) was added to enhance differentiation towards NK-lineage.

For transfer to Rag2^{-/-}γC^{-/-} mice, differentiated hematopoietic cells were harvested as described above, after which dead cells, debris, and remaining TSt-4 cells were removed with a FACS Aria. Purity of sorted cells was 80-90%.

Polymerase chain reaction analysis

Genomic DNA was extracted from cells using phenol-chloroform according to standard procedures. Primers and positive controls used for detection of TCR rearrangements are described in Dik *et al.*(11). PCR was carried out in 25 µl reaction volumes containing either 50 ng DNA from differentiating cells or the negative control (breast cancer cell line ZR75-1, kindly provided by the Department of Pathology, MUMC⁺), or 1 ng positive control DNA. All PCR components were used according to the manufacturer's instructions (iTaq, BioRad, Hercules, CA), supplemented with 1× bovine serum albumine (New England Biolabs, MA) and 540 nM of each primer (Eurogentec, Liege, Belgium). Optimal MgCl₂ and anneal-temperature for each primer-set proved to be 5mM/63.9°C and 3mM/60.5°C for Dδ2–δ1 and Vδ1–Jδ1, respectively. All PCR products were validated by TA-cloning and sequencing according to standard procedures.

Cytotoxicity assay

Target cells (K562) were labeled with 3'-diiododecylcarbocyanine (DiO) according to the manufacturer's instructions (Sigma). 2×10^4 Target cells were incubated with effectors at various effector:target (E:T) ratios for 4 hours, each ratio in triplicate. Percentages of killed target cells (PI⁺ DiO positive) were determined by flowcytometry. Percentages

specific lysis were calculated as follows: ((% PI positive target cell/% spontaneous PI positive cells)/ % of vital cells) $\times$ 100.

Rag2^{-/-}γc^{-/-} mice

Rag2^{-/-}γc^{-/-} mice on a BALB/c background (kindly provided by Dr. M. Ito and the Central Institute for Experimental Animals, Kawasaki, Japan) were bred and maintained under specific pathogen-free conditions in accordance with the guidelines of the animal facility at the Institute for Research in Biomedicine.

At day of birth, newborn Rag2^{-/-}γc^{-/-} mice were irradiated in a 4 hour interval with 2 times 1.25 Gy (Rad Source Technologies, Inc., Alpharetta, GA) at 1 Gy/min, a dose that was titrated to be sublethal. At 4 hours post irradiation, mice were transplanted with either 1.5×10^5 CB-CD34⁺ cells, 1.5×10^6 mCD34⁺ cells or 3×10^6 T/NK progenitors in 25 μl PBS into the liver (i.h.) using a 30-gauge needle (Hamilton Bonaduz AG, Bonaduz, Switzerland), as described previously (12). Mice were weaned at 3 weeks of age.

Results and discussion

mCD34⁺ cells develop into T/NK-lineage progenitors on hDLL1/4 interaction

Sorted mCD34⁺ cells were cultured on monolayers of TSt-4 cells expressing either hDLL1 or hDLL4, and on TSt-4 control monolayers (Supporting Information Figure 1). First, we analyzed the time-dependent development of the phenotypic and genotypic markers associated with T-lineage development in human thymus (11, 13). Intracellular CD3⁺ (iCD3) cells became detectable after 1 week of coculture, expanded strongly during the following weeks, and costained with T-lineage-associated surface markers (Figure 1A; Supporting Information Figure 2). After 4 weeks, the overall phenotype and percentage distribution of the cells had stabilized, similar to the differentiation kinetics described for CB-CD34⁺ cells on TSt-4-hDLL1(9). Within this time period, cell numbers had increased 200 to 600 times (Figure 1B) with on average 50% of the cells coexpressing iCD3, CD45RA, CD7, and CD5 (Figure 1A). Differences in overall phenotype of the cell populations were minimal

between different mCD34⁺ cell donors, or whether TSt-4-hDLL1 or 4 was used to induce differentiation (Figure 1A).

To confirm T-lineage commitment, we analyzed early and late recombination events of TCR genes, D δ -J δ and V δ -J δ /D β -J β , respectively (11). After 4 weeks, T-lineage progenitors from all donors analyzed had either partially or completely rearranged the D δ 2-J δ 1 genes, whereas a smaller proportion had continued with rearrangement of the V δ -J δ genes (Figure 1C). There were no rearrangements of D β -J β genes detected (not shown).

Figure 1. mCD34⁺ cells develop into T/NK progenitors on DLL1/4 interaction. mCD34⁺ cells were obtained from 3 donors, V1 to V3. (A) Expression of indicated T-lineage-associated markers has stabilized after 4 weeks of culture on TSt-4-hDLL1/4 monolayers. For V2 and V3, the population percentage distribution of each marker combination falls within the following ranges: 88% to 92% for CD45RA⁺iCD3⁺ and 45% to 55% CD7⁺CD5⁺. For V1, these percentages are 63% to 68% and 37% to 58%, respectively. (B) Numbers/donor represent minimum and maximum of fold increases observed in 2 or 3 independent experiments/donor. (C) DNA was isolated from cells cultured on TSt-4 and TSt-4-hDLL1/4 for polymerase chain reaction analysis of gene rearrangements at the TCR locus. At the δ -locus, all donors show clear D δ -J δ rearrangement products, whereas V δ -J δ rearrangement products are faint and therefore must have occurred less frequently. In contrast to positive controls, 2 products are amplified from DNA of mCD34⁺ cell-derived progenitors. Sequence analysis demonstrated that the large products (> 1000 bp) are partial rearrangements of the δ -locus. (D) When differentiated cells were cultured for another 4 weeks on TSt-4 without DLL, the population lost surface expression of the T-lineage-associated marker CD5.

Figure 1. mCD34⁺ cells develop into T/NK progenitors on DLL1/4 interaction (continued).

(E) Instead, cells gained expression of the NK lineage-associated markers NKp46 and NKG2A but did not express the killer cell immunoglobulin-like receptor proteins CD158a-c. Again, there are no differences between hDLL1- or hDLL4-derived cells. Similar results were obtained with another donor. (F) TSt-4-derived cells have killing capacity when cocultured at indicated effector (e) target (t) ratios with the HLA class I-negative leukemia cell line K562. Each point represents the average of a triplicate analysis. Similar results were obtained with another donor. Purified peripheral blood (PB) NK cells from healthy controls always served as positive control.

Both phenotype and genotype of the T-lineage directed cells indicated that they were arrested at the T/NK pre-T-cell stages of T-lineage development (11, 13-15). Indeed, on transfer to TSt-4 control monolayers, cells gradually lost the T-lineage-associated marker CD5 (Figure 1D) and gained expression of the NK lineage-associated markers NKp46 and NKG2A (Figure 1E). Furthermore, the fact that the cells were able to kill the leukemia cell line K562 (Figure 1F) and did not express killer cell immunoglobulin-like receptor ligands (Figure 1E) established that they had developed into immature NK cells (16).

T/NK progenitors fully mature on transfer to Rag2^{-/-}γC^{-/-} mice

To determine whether these T/NK progenitors were able to complete their maturation in vivo, adoptive transfer experiments were performed using Rag2^{-/-}γC^{-/-} mice. Figure 2A shows that human T/NK progenitors home to the thymus of these mice and become

CD4⁺CD8⁺ double-positive (DP) thymocytes within 5 weeks after transfer. Furthermore, a clear population of surface CD3 and TCR- $\alpha\beta$ -expressing thymocytes was observed within 7 weeks (Figure 2A). Along with the appearance of TCR- $\alpha\beta$ ⁺ cells, there was a concomitant decrease in total number of huCD45⁺ cells in these thymuses (Figure 2A). This demonstrates that the T/NK progenitors are able to complete their TCR gene rearrangements and implicates that a single shot of T/NK progenitors provides one spatiotemporal wave of developing thymocytes. No CD56 expression was detected in the thymus (Supporting Information Figure 3), nor could huCD45⁺ cells be found outside of the thymus in mice given T/NK progenitors (Figure 2C), indicating that the lineage development was restricted to T cells in Rag2^{-/-} γ c^{-/-} mice.

As mentioned, in T/NK progenitor-injected mice, all huCD45⁺ cells were CD4⁺CD8⁺ DP by week 5, in contrast to animals that had received either CB- or mCD34⁺ cells. In these latter groups, T-lineage development was delayed by approximately 2 weeks, as percentage DP along with total number of huCD45⁺ cells at week 7 corresponded with week 5 data of mice supplied with T/NK progenitors (Figure 2B; Supporting information Figure 4), confirming the advantage provided by in vitro DLL-mediated predifferentiation.

When compared, there were not many huCD45⁺ cells left in bone marrow at week 7 in mCD34⁺ cell-transplanted mice (Figure 2C). Apparently, the long-term engraftment potential of CB is favorable over mCD34⁺ cells in Rag2^{-/-} γ c^{-/-} mice.

At 7 weeks, one mouse given hDLL4 T/NK progenitors had very few huCD45 cells left in the thymus (Figure 2B-C), and none had mature T cells in the periphery. This is not unexpected because more than 95% of the thymocytes do not survive the positive/negative selection process, and the underdeveloped thymuses of Rag2^{-/-} γ c^{-/-} mice have considerably reduced capacity. Even in CB-CD34⁺ cell-transplanted mice it requires continuous immigration of pro-thymocytes for 8 to 10 weeks before mature T cells can be found outside the thymus (12).

The most important finding of this study is that mCD34⁺ cell-derived, in vitro-generated T/NK progenitors are able to migrate to the thymus and continue their development toward mature T cells. Conceptual similar findings were recently described for CB-derived progenitors (17). Prospectively, cotransplantation or sequential transplantation of CD34⁺ cells and T/NK progenitors will narrow the immunocompromised window of HSCT patients

considerably. Furthermore, the donor-derived thymus-matured T cells will also contain a patient-tolerant regulatory T-cell repertoire that may reduce graft-versus-host disease–related complications.

Figure 2. T/NK progenitors fully mature on transfer to Rag2^{-/-}γc^{-/-} mice.

mCD34⁺ were differentiated on monolayers of TSt-4 expressing hDLL for 4 weeks, after which they were purified by cell sorting and injected intrahepatically into newborn Rag2^{-/-}γc^{-/-} mice. (A) Both hDLL1- and hDLL4-derived T/NK progenitors are found exclusively in the thymus at both time points analyzed and have progressed toward the CD4⁺CD8⁺ DP stage at 4.5 weeks. A limited number of DP and few CD8 single-positive (SP) cells already have CD3 surface expression (Supporting information Figures 3–4). The CD4⁺ cells at this time point are immature single-positive cells (ISP); sCD3⁻ and CD5⁺CD7⁺. At 6.5 weeks, the percentages of huCD45⁺ have dropped sharply, but there is clear surface expression of sCD3 and TCR-αβ on T/NK progenitor-derived cells, on both DP and CD4/CD8 SP cells (Supporting information Figure 4).

Figure 2. T/NK progenitors fully mature on transfer to Rag2^{-/-}γ_c^{-/-} mice (continued).
(B-C) Next to T/NK progenitors, mice were given mCD34⁺ or CB-CD34⁺ cells. (B) Thymus compartment was analyzed at indicated time points. Thymocyte subsetting is based on coexpression of CD5 and CD7 only (double-negative), CD4⁺sCD3⁻ (ISP), CD4⁺CD8⁺ (DP), and CD4⁺sCD3⁺ or CD8⁺sCD3⁺ (SP). The left 2 bars of each graph represent the data from mice displayed in panel A. Compared with mCD34⁺ and CB-CD34⁺ cells, predifferentiation of mCD34⁺ cells results in a higher percentage of cells at the DP stage at 4.5 weeks, but few cells are left at 6.5 weeks. (C) The total number of huCD45⁺ cells/tissue shows that T/NK progenitors are present temporarily and exclusively in the thymus.

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Supporting information

Figure 1. Set-up of mCD34⁺ cell differentiation on TSt-4 and TSt4-hDLL monolayers.

mCD34⁺ cells were seeded at a density of 2.6×10^3 cells/cm² on monolayers of TSt-4 cells expressing Notch ligands DLL1 or DLL4. During the 1st week, cultures were maintained in medium containing 10% FBS, upplemented with 100 ng/ml SCF, 100 ng/ml Flt3-L, 50 ng/ml TPO and 10ng/ml IL-7. After 1 week, co-cultures were maintained in complete medium supplemented with IL-7 (20 ng/ml). Co-cultures were refreshed by halve medium change 3× weekly. Differentiating HCs were transferred to fresh monolayers every 2 weeks.

Figure 2. Time-dependent development of DLL-induced T-lineage associated markers on mCD34⁺ cells. CD7 and iCD3 are differentiation-markers associated with T/NK cell lineage commitment that are upregulated during the first 4 weeks of culture. Results for mCD34⁺ cell donor V1 on Tst-4 and Tst-4-hDLL1 are shown. Quadrants are based on iCD3 background-staining of mCD34⁺ cells expanded on Tst-4.

Figure 3. CD56 expression on huCD45⁺ cells in thymuses of Rag2^{-/-}γc^{-/-} mice. Human CD45⁺ cells were stained for CD56 (and CD3). There may be insufficient production of growth factors (like IL-15) that are required for normal development of NK cells in mice reconstituted with T/NK progenitors.

Figure 4. Expression of sCD3 on thymocyte subsets in Rag2^{-/-}γC^{-/-} mice. Human CD45⁺ cells were stained for CD4 and CD8, and quadrants were set for thymocyte-subsets indicated in the legend. Histograms display relative levels of surface CD3 expression of the subsets in each quadrant. CD3⁺CD4⁺ and CD3⁺CD8⁺ cells derived from T/NK progenitors were detected at both time-points. At week 6½, the results of the second DLL4 T/NK progenitor-injected mouse are difficult to interpret. Because of the low %CD45 left in the thymus (0.4%), it was not possible to do an additional staining for CD5 and CD7. Therefore we do not know if the CD4⁺ cells displayed are bona fide ISP. The fact that all other T/NK progenitor injected mice already had DP thymocytes at week 4½, it is likely that negative selection deleted most thymocytes and one is left with ISP-stage arrested cells.

Chapter

8

General discussion

Immunotherapy for cancer: No one man show!

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NK cells: The secret weapon in DC vaccination strategies*

Introduction

In the early 1900s, the concept of involvement of the immune system in cancer development was first postulated by Ehrlich (1) and later transformed into the hypothesis of tumor immunosurveillance by Burnet and Thomas (2, 3). However, it took until the 1990s before it was shown in mouse models that the hypothesis indeed was true (4). Immune competent mice were namely able to reject transplantable tumors. Eventually, due to increasing evidence, tumor immunosurveillance was acknowledged by many clinicians and translated into therapeutic concepts (5). Cancer patients were treated with either cancer cell vaccination, inoculation of serum prepared against a tumor or bacterial vaccines. Successes were reported, however, due to poor experimental execution clinical effects were unclear. Later, also donor bone marrow stem cell transplantation was explored making use of a donor's immune system to fight cancer. Thus far, this is the most effective immunotherapeutic method used in patient care, though also here success is still limited.

Increasing knowledge about the key players of the immune system involved in cancer like B cells, natural killer (NK) cells, antigen presenting cells (APC), T helper (T_H) cells, cytotoxic T cells (CTL) and regulatory T cells (Tregs) combined with the knowledge of interactions between these cells, have led to the development of improved immunotherapeutic strategies to fight cancer (6). While understanding more and more about the immune system also its complexity became cognizant. This led to a very broad field where immunologists tried to focus on only one cell type or one mechanistic system to cure cancer, without studying the complete immune response. This focus on single immune cells is probably why we became very efficient in eliminating cancer in genetically engineered mice in which only a very specific part of the immune system is studied, but experience many difficulties when conducting human trials (7, 8). When the body has such a sophisticated and well designed immune system that harnesses it especially against infections, it seems logical that inducing activation of the whole system in the fight against cancer would be more effective than focusing on single elements.

Evidence for the strength of cooperation in anticancer therapy is illustrated by conventional therapeutic options, which are mainly based on the combination of surgery,

chemotherapy, hormonotherapy and radiation. Over the past decades several different combinations of chemotherapy have been utilized, combining alkylising cytostatics, antimetabolites, antimitotic agents, antitumor antibiotics, topo-isomerase inhibitors and others (9). These facts on anticancer therapy underline the need for combination therapy to attack tumors by different mechanisms.

This has now been acknowledged by many researchers who are convinced that the immune system is such a strong defense mechanism due to the cooperation of different immune players (10). Therefore, efforts have been made in combining either different immunotherapeutic strategies, or combining immunotherapy with conventional anticancer therapy.

In this thesis, we have investigated and optimized different immunotherapeutic strategies *in vitro*, including tumor-specific antibody transfer, induction of T and NK cell responses by DC vaccination and adoptive transfer of pre-T cells. The rationale behind this is that we are convinced that the complete immune system should be activated to induce effective anticancer responses.

This discussion will explain the rationale behind combination therapy, which will be illustrated by the effect of DC vaccination on NK cell activation. Additionally, other therapeutic approaches that have been reported effective in combination with DC vaccination, will be reviewed.

Clinical DC vaccination strategies

Clinical DC vaccination trials illustrate nicely the downside of focusing on only one immune reaction, rather than studying the effects on the whole immune system. The initial vaccination strategies focused on inducing anticancer CTLs (11). However, after unraveling the function of MHC class II and the identification of T_H cells, the induction of CTLs was analyzed in the context of T_H cell polarization (12, 13). This eventually led to the development of DC differentiation and maturation cocktails that enhance the induction of T_H1 polarization and the use of readout systems to evaluate the induction of these responses *in vitro* and *in vivo* (14). Even though the focus was not only on CTL induction

but also on T_H cells, activation of other cell types was not acknowledged. In clinical DC vaccination strategies, about 10-20% of patients respond to therapy by tumor regression or stable disease, sometimes even complete remission is reported (15). However, these clinical outcomes often very poorly correspond to the induction of T_H1 cells or tumor-specific CTLs (Table I). This means either that T cell responses are analyzed at the wrong anatomical site or that other non-T cell responses are induced, which are also successful in anticancer reactivity.

Next to T cell responses, sometimes also B cell responses were evaluated, by detection of tumor-specific antibodies. Though, results from clinical vaccination studies show that these tumor-specific antibodies are often produced after vaccination, they correspond very poorly with clinical responses (16). It took until 2000, before also other effector cells were analyzed in the context of DC vaccination. Martin-Fontecha et al. showed in mice that NK cells become activated after injection of matured DCs (17). They even showed that NK cells are an absolute requirement for the induction of T_H1 polarization. But what about human studies? As nicely reviewed by Moretta, human *in vitro* data demonstrate that NK cells and DCs cooperate in pathologic conditions (18). Triggered by these human data, NK cell responses were evaluated in clinical DC vaccination studies. In a trial in AML patients, Van Tendeloo et al. showed that there was a significant correlation between clinical responses and the presence of high numbers of activated (HLA-DR⁺) NK cells post-vaccination (19). Additionally, other studies in patients with solid tumors showed comparable correlations (Table II). In these studies, NK cell activation was often analyzed phenotypically, by increased expression of activation markers, or functionally by an enhanced cytotoxic or cytokine-producing capacity (20).

The immunostimulatory effect of NK-DC interaction is based on a bidirectional crosstalk by which both cell types can become activated by either contact-dependent or independent factors. For DC vaccination techniques, activation of both cell types *in vitro* has been described and their effect on other immune cells and tumor cells has been suggested beneficial for the induction of antitumor responses (21, 22).

Table I. T cell activation in clinical DC vaccination trials

Disease	Vaccination	Clinical response	Specific T cells	Reference
Acute myeloid leukemia	WT1 mRNA-electroporated DCs	5/10 patients (2/10 with long lasting responses)	2/5 patients Best T cell responses in best clinical responders	(19)
	Wild type and modifies gp100 peptide-pulsed DCs	9/27 patients (1/27 complete response)	3/27 patients Best T cell responses in best clinical responders	(23)
Breast cancer	MUC1 gene transfected DCs.	1/10 patients	4/10 patients	(24)
Renal cell cancer	DC vaccination with tumor cell lysate or tumor cell line lysate	10/27 patients (2/27 complete response)	5/6 patients. Best T cell responses in best clinical responders	(25)
Hepatocellular carcinoma	DC vaccination with tumor cell lysate	7/25 patients	5/10 patients no Correlation with clinical responses	(26)
Melanoma	Tumor antigen cocktail loaded DCs	11/36 patients	6/10 no correlation with clinical responses	(27)
Multiple myeloma	Idiotypic-pulsed DCs	5/9 patients	4/5 patients that had a clinical	(28)
Colorectal cancer	Tumor lysate loaded DCs	8/24 patients are disease free (significantly better survival when specific T cell response)	15/24 patients	(29)

Table II. NK cell activation in clinical DC vaccination trials.

Disease	NK cell responses	P-value	Reference
Acute myeloid leukemia	4/5 clinical responders 0/5 nonresponders	0.01	(19)
CEA positive tumors (colorectal, lung and urachal cancer)	4/5 clinical responders 0/4 nonresponders	0.016	(30)
Non-Hodgkin lymphoma	5/6 clinical responders 1/4 nonresponders	0.009	(31)

NK cell-dependent DC responses

NK cells hold the capacity to control and enhance DC-mediated antitumor immune responses. They do this by inducing maturation op T_H1-polarizing DCs and killing of inappropriately matured DCs. Additionally the NK cell’s intrinsic capacity to kill malignantly transformed cells, provides DCs with antigenic material for presentation.

NK cell-induced DC maturation

NK cells have been shown to mediate immunoregulatory “helper” functions. These helper NK cells were defined by Mailliard et al. as CD83⁺CCR7⁺CD56^{dim} NK cells that possess a DC-activating capacity (22). This DC activation is mediated by both soluble factors produced by NK cells as well as ligation of DC-surface receptors with activating ligands expressed on the NK cell surface (Figure 1A). Important soluble NK cell-derived factors implicated in NK-DC crosstalk are INF- γ and TNF- α (32-34). The production of these cytokines by NK cells depends on reciprocal DC-induced NK cell activation by engagement of the NKp30 receptor (35, 36). NK-DC interaction results in the development of stable, type-1 polarized DCs that produce high amounts of proinflammatory cytokines and thereby hold the capacity to enhance T_H1 and CTL-mediated immunity against intracellular pathogens and cancer (Figure 1A) (32, 37, 38). Additionally, human *in vitro* and mouse *in vivo* models

have shown that the production of IFN- γ and TNF- α by NK cells is also directly associated with induction of enhanced antitumor T_H1 cell responses (17, 35, 39, 40), whereas in the absence of NK cells, T_H polarization is biased toward T_H2 polarization, without induction of CTLs (41). NK cell-derived IFN- γ induces upregulation of the major T_H1 transcription factor T-bet and inhibition of the T_H2 transcription factor GATA-3 (17, 22). These IFN- γ -secreting cells were named “helper” NK cells, which gained their function after activation by a combination of IL-18 and IL-12 (22). Next to IL-12 and IL-18, also other pro-inflammatory cytokines (IL-2, IL-15, Type-I interferons: IFN- α/β), NK-cell sensitive tumor cell lines and opsonizing tumor-specific antibodies can induce NK cell activation and IFN- γ production (42, 43). However, NK cell activation depends on a two-signal mechanism where two activating signals have to synergize (38, 44). Recently, it was shown that NK cells from melanoma patients can efficiently fulfill their “helper” function *in vitro* after induction with IL-18 and IFN- α (42). These NK cells are high IFN- γ producers and induce a DC subset which is superior at inducing antigen specific T cells as compared to non-polarized standard DCs. Whether these “helper” NK cells also naturally occur *in vivo* and whether they indeed represent a distinct NK cell subpopulation is still unknown. Reports on the IFN- γ secreting NK cell subset are controversial. The CD56^{bright} NK cell subset was historically described as the cytokine-secreting subpopulation (45), however, others including us, showed that the CD56^{dim} population can also produce large amounts of IFN- γ after NK-DC interaction (21, 22). Our studies on NK cell phenotype, show that NK cells display a high plasticity and upon stimulation can easily up- and downregulate surface receptor expression (unpublished data). Therefore, we propose that NK cell function and phenotype depend on the direct micro-environment and NK-induced DC maturation is not confined to a certain subtype of NK cells.

Figure 1. NK cell-dependent DC activation.

NK cells can enhance DC function by three different mechanisms. (A) NK cells can become activated to produce IFN- γ and TNF- α by a two-signal mechanism, including activation by cytokines (IL-12, IL-18, IL-15, IL-2 and IFN- α/β), by induction of ADCC via antibodies or by ligation of activating receptors (NKp30, DNAM-1). Both NK cell-derived cytokines enhance maturation of IL-12-producing DCs, which effectively induce T_H1 polarization. Additionally, NK cell-derived IFN- γ directly enhance T_H1 polarization. (B) Due to NK cell-mediated tumor cell lysis, tumor antigens become available for DCs to engulf and present. Activated NK cell also upregulate the expression of MHC class II and become weak antigen presenters themselves. (C) NK cells kill immature DCs (iDCs), because of low HLA-E expression. The ligation NKp30 enhances this NK cell function. By elimination of iDCs, NK cells prevent T cells to become tolerogenic due to contact with inadequately matured DCs.

Facilitation of antigen presentation

Next to their “helper” effect on DC activation, NK cells have the natural capacity to kill virally infected or malignantly transformed cells (46). This function is not only of direct use for tumor elimination, but also facilitates uptake and cross-presentation of tumor-related antigenic material by DCs (47, 48). Thereby, NK cells contribute to DC-mediated induction of tumor-specific T cells (Figure 1B). Additionally, activated NK cells are induced to express MHC class II and costimulatory molecules (CD80, CD83 and CD86) and are therefore also able to present antigen to T cells themselves (Figure 1B) (49-51). These NK cells with DC-like qualities have been described in humans and mice and have been reported as a new immune cell subpopulation referred to as NKDCs as well as IKDCs (IFN-producing killer dendritic cells) (52-57). Though their etiology is not yet completely elucidated, IKDCs are functionally and developmentally more similar to NK cells than to DCs (58-60). MHC class II expressing-NK cells are able to present antigen and induce T cell responses, however, conventional DCs are much more efficient antigen presenters. Target cell lysis and cytokine secretion of IKDCs is comparable to conventional NK cell function. Developmentally, mouse IKDCs resemble activated, human, MHC class II expressing NK cells, and are absent from recombinaise activating gene-2-null, common γ -chain-null (RAG2-/-IL2rg-/-) mice, which lack NK cells but not DCs. Additionally, these cells efficiently proliferate by IL-15 activation (58, 61). Although nomenclature might be contradictory and confusing, these different subsets are most likely to resemble activated NK cells (52). In summary, by their natural cytotoxic capacity NK cells provide DCs with antigenic material. These antigens can be presented by DCs as well as by NK cells to induce effective adaptive antitumor responses.

NK cell-dependent lysis of immature DCs

NK cells are not only able to kill tumor cells, but also have the capacity to eliminate both autologous and allogeneic immature DCs (iDC). NKp30, which is involved in NK cell-mediated DC maturation, has been shown to play a major role in iDC lysis (32) and cooperates with DNAM-1, a triggering receptor expressed by virtually all NK cells (62) (Figure 1C). Since both, mature as well as immature DCs express ligands for NKp30 and DNAM-1 (62, 63) an additional mechanism must be involved in the recognition of iDCs by

NK cells. Analysis of NK cell clones has revealed that killing of iDCs was confined to NK cells that lack expression of inhibitory KIRs specific for self-HLA class I alleles, but do express the HLA-E-specific CD94/NKG2A inhibitory receptor (64). The iDC becomes susceptible to NK cell-mediated cytotoxicity, not because of low expression of all HLA molecules, but only because of low expression of HLA-E. In contrast, mature DCs express higher amounts of HLA-E and are therefore kept untouched (64, 65). Upregulation of MHC and costimulatory molecules is needed for DCs to induce efficient T cell responses. Whereas mature DCs are thought to be the most effective antigen presenters and inducers of adaptive immunity, iDCs have been implicated in tolerance and induction of regulatory T cells (66). Thus, it can be hypothesized that by elimination of iDCs, NK cells ensure the activation of adaptive immune responses by preventing inadequately matured DCs to interact with T cells (67). Interestingly, in an immunosuppressive environment with high production of IL-10, it has been demonstrated that iDCs become less susceptible to NK cell-mediated lysis, whereas mature DCs exhibited increased susceptibility to NK cell-dependent elimination (68). Though in apparent paradox, NK cells can induce DC maturation as well lysis of iDCs. This paradox can be explained by work from Piccioli et al., who showed that the result of NK cell activation depends on the relative numbers of each cell type and NK cell-mediated lysis of iDCs is only induced if NK cells outnumber iDCs (32).

In conclusion, NK cells induce effective antitumor immune responses, by inducing DC maturation and antigen presentation, but inhibiting tolerogenic responses mediated by inappropriately matured DCs.

DC-induced NK cell responses

The bidirectional crosstalk between NK cells and DCs, is also characterized by DC-mediated NK cell activation. DCs are capable of providing NK cells with soluble as well as contact-dependent activators, thereby enhancing their cytokine production, proliferation, survival and cytotoxicity. NK cells make use of all these four mechanisms in to eliminate cancer cells.

Induction of cytokine production and proliferation

Reciprocally, DCs activate NK cells to produce cytokines. Also this activation is mediated by a two signal mechanism of which both signals are DC-derived. The major cytokines produced by NK cells after NK-DC crosstalk are $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$. NK cell activation by DCs is mediated by both soluble as well as contact-dependent factors (Figure 2). Depending on their maturation stimuli DCs upregulate the expression of NK cell- activating surface molecules and start producing NK cell-stimulating cytokines (59, 60).

Figure 2. DC-induced NK cell activation.

DCs can affect NK cell function by augmentation of cytotoxicity, cytokine secretion ($\text{IFN-}\gamma$ and $\text{TNF-}\alpha$) and proliferation. This depends on contact-dependent (ligation of NKp46, NKp30, NKG2D, 2B4 and CD27 and presentation of IL-15 *in trans*) as well as soluble factors (IL-2, IL-12, IL-15, IL-18, IFN- α and IFN- β). IFN- γ secretion by NK cells is in turn responsible for DC maturation and T_H1 polarization, whereas augmentation of NK cell cytotoxicity contributes to tumor cell lysis. Which cytokines or contact-dependent factors are involved depends on the triggers involved in DC activation (most likely TLR ligands).

Among the soluble factors IL-12 and IL-18 have shown highly efficient in NK cell activation. We and others have shown that this capacity is confined to DCs triggered by PRR (pattern recognition receptor)-signaling, which produce high amounts of IL-12 and IL-18 (11, 61). Both cytokines synergize in the induction of NK cell activation, which requires the formation of an immunological synapse with cytokine secretion polarized at the NK-DC interface (62, 63). Additionally, it has been demonstrated in humans and mice that DC-derived IL-2 also contributes to NK cell activation (64, 65). IL-15 is another DC-derived factor, which works both as a soluble factor as well as a contact-dependent factor (10, 66, 67), since DCs as well as DC exosomes can bind IL-15 to their IL-15R α thereby presenting IL-15 *in trans*. The effect of IL-15 on NK cell proliferation, survival and cytokine secretion is much stronger, when presented *in trans* than in its soluble form (66).

In addition to these soluble factors, it was shown that NK-DC crosstalk was enhanced when both cell types were in contact with each other, suggesting that also contact-dependent factors must be involved in this crosstalk (22). These factors include surface molecules expressed on DCs that ligate with activating NK cell receptors. One of these NK cell receptors is NKG2D, which is an C-type lectin coactivation receptor expressed on all NK cells (68). NKG2D binds to stress-inducible members of the polymorphic MHC class I-related Chain A/B (MICA/MICB) family, which are upregulated on mature DCs (69, 70). The NK cell activating receptors Nkp30 and Nkp46 are involved in NK-DC crosstalk as well as in NK-mediated tumor cell lysis (25, 71, 72). In mice also CD27, expressed by NK cells, has shown to be implicated in NK-DC crosstalk. It binds to the co-stimulatory molecule CD70, which is exclusively expressed by DCs and therefore the CD27^{high} expressing mouse NK cell subset is superior in cytokine production after DC interaction. Since this subset resides in the lymph nodes, it has been proposed that these cells might resemble the human CD56^{bright} NK cell subset (73, 74). Moreover, 2B4, which ligates with CD48, has been implicated in NK-DC crosstalk however, its function is still promiscuous. The 2B4 pathway can be both activating as well as inhibitory (75, 76). Which pathway is induced depends on the NK cell maturation status and on NK cell localization (77). It has been demonstrated by Morandi et al., that lymphoid NK cells express 2B4 that functions as an inhibitory receptor. However, they also show that lymphoid DCs show very low expression of CD48, thereby preventing NK cell inhibition (78). All the previously mentioned surface molecules are

stress-dependent factors that can be upregulated during DC maturation, thereby mediating danger signals to the surrounding tissue.

Taken together these studies indicate that NK cell activation results from a combination of DC-derived factors. These factors include soluble as well as contact-dependent stimuli. Which mechanism is utilized depends on the DC maturation status. NK cell activation involves cytokine production, cytotoxicity, proliferation and increase of their lifespan. Production of IFN- γ by NK cells in response to DC activation is again involved in T_H1 polarization and thereby induction of CTLs (7).

Induction of NK-mediated cytotoxicity

NK cells can lyse virally infected or malignantly transformed cells. This cytotoxic activity is based on a delicate balance of inhibitory and activating signals and depends on the expression of MHC class I and activating ligands on the target cell as well as on expression of the respective receptors by NK cells (89, 90). NK cell cytotoxicity can be enhanced by DC-dependent mechanisms (Figure 2) (33, 91). These mechanisms include DC-derived IL-12 (21), IL-2 and IL-15 (92) as well as contact-dependent factors like NKG2D, NKp46 and NKp30 (81). Which cytokines or NK cell receptor ligands are responsible for the enhanced tumor cell lysis depends on different DC subsets or maturation stimuli (92, 93).

Common playground for NK cells and DCs?

For reciprocal NK-DC activation it is obligatory for the two cell types to meet. It has been proposed that NK-DC interaction takes place at the site of inflammation as well as in the lymph nodes (18). During an inflammatory response, a burst of immunoactive molecules is secreted including chemokines. These chemokines are responsible for the recruitment of immune cells, which produce even more chemokines and cytokines in response to the inflammatory reaction. For NK-DC crosstalk specifically, it is necessary that one cell type produces chemokines, important for recruitment of the other cell type. We have shown that upon maturation TLR-triggered DCs produce high quantities of many different chemokines (94). Among these, there are NK cell recruiting chemokines produced like

CCL5, CXCL10 and CCL19. We have shown that in human TLR2/4 matured DCs, the most important chemokine receptor for NK cell recruitment is CCR5 (21). However, other groups have also reported on the involvement of CXCR3 and its subsequent chemokines CXCL10 in recruitment of blood-derived NK cell by LPS and poly I:C triggered DCs (17, 71). Therefore, it seems that there are multiple, non-redundant mechanisms to recruit NK cells. Which of these mechanism is utilized depends on the DC maturation-triggers and eventually on the invading microorganism.

Figure 3. Where do NK cells and DCs interact?

Tissue-resident DCs matured upon inflammatory stimuli produced by the tumor and its surrounding tissue. Upon maturation, these DCs upregulate MHC, costimulatory molecules and CCR7. Additionally, these DCs start to produce cytokines and chemokines. These chemokines are responsible for NK cell recruitment (either CCR5 or CXCR3-dependent). (A) Blood-derived NK cells are then recruited into the tumor environment and interact with DCs resulting in reciprocal activation of both cell types. Upon maturation, DCs upregulate their CCR7 expression and migrate into the draining lymph nodes. (B) While migrating, DCs still produce chemokines and thereby recruit NK cells into the lymph nodes. (C) Additionally, NK-DC interaction induces the upregulation of CCR7 expression by NK cells, which constitutes a secondary mechanism by which NK cells migrate into the draining lymph node. (D) Due to migration of both cell types into the lymph node and also the presence of lymph node-residing NK cells, NK-DC crosstalk can take place in the lymph node.

Since maturing DCs migrate into the draining lymph nodes, chemokine production by mature DCs is possibly also responsible for the recruitment of NK. It has been shown in CXCR3^{-/-} mice that T_H1 polarization was inhibited, due to impaired recruitment of NK cells into the lymph nodes (17). In humans, however, additional NK cell-migrating mechanisms have been proposed. NK cells are able to upregulate their CCR7 expression and subsequently their responsiveness to the lymph node-associated chemokine CCL19 upon activation with IL-18 and upon NK-DC crosstalk (21, 22). This would represent a secondary mechanism by which DCs induce NK cell migration into the draining lymph nodes. These already activated NK cells could effectively induce T_H1 polarization (Figure 3).

In summary, DCs induce NK cell migration either due to recruitment by DC-derived chemokines and/or due to upregulation of CCR7 after NK-DC crosstalk, thereby facilitating interaction of the two cell types in the lymph node or at the site of inflammation.

NK-DC crosstalk in immunotherapy

The reciprocal effects of NK-DC interaction provide a strong rationale for the combined use of NK cells and DCs in immunotherapy. Autologous as well as allogeneic NK cells have been used in cancer immunotherapy and have been proven effective in defined tumors (95). However, in these therapies, NK cells were always used as lonely killers. With our recent knowledge about NK-DC interaction, one could argue that DCs used in DC vaccination strategies should be matured by NK cells, since these DCs produce high concentrations of IL-12 and are therefore excellent T_H1 polarizing DCs (42). When looking from another point of view, there is also a rationale to use DCs that are able to optimally activate and recruit NK cells (21, 71). The NK cell-activating capacity of these DCs depends on the maturation triggers used for *ex vivo* DC maturation. Additionally, an absolute prerequisite for these DCs is that they actively recruit NK cells (17, 21). We and others have shown that for this purpose DC maturation protocols should contain TLR-triggering agents whereas PGE₂, another DC activating molecule produced during inflammation, has a negative effect on the production of NK cell-activating cytokines and NK cell-recruiting chemokines (71). These results are in line with data showing that PGE₂ matured DCs

induce more T_H2 than T_H1 polarization and induce a lower number of tumor-specific CTLs (14, 94, 96). Even though these reports on the negative effects of PGE2-mediated DC maturation, most clinical trials use PGE2-matured DCs. The rationale behind this is the *in vitro* capacity of these DCs to migrate towards a CCL19 gradient (97, 98). Therefore, PGE2-matured DCs are also thought to migrate into the lymph nodes after injection *in vivo*. However, *in vivo* studies do not support this hypothesis. TLR-matured DCs are not only superior in the induction of T_H1 polarization but are also much better NK cell activators by inducing both NK cell-mediated cytotoxicity and IFN- γ production, resulting in strong antitumor responses. The lack of migratory capacity of TLR-matured DCs opens the possibility for the combination of NK-DC therapy, where both cell types are injected into the tumor. Thereby, both cell types will be directly present at the battlefield and are able to interact. NK cells lyse tumor cells, thereby making antigenic material available for DCs to take up and present. Additionally, NK cells induce maturation of bystander DCs and lyse inappropriately matured iDCs. Reciprocally, mature DCs can activate NK cells to proliferate, secrete cytokines and augment NK cell cytotoxicity.

All three options for the combined use of NK cells and DCs in vaccination strategies for cancer are based on theoretical solid grounds. However, only the activation of NK cells by DC vaccination strategies has been utilized and shows promising results (19).

Tertiary lymph node

As mentioned before, *ex vivo* matured DCs show a bad migratory capacity *in vitro*. The positive effect of PGE2 on *in vitro* DC migration is promising (97, 98), *in vivo* migration of these DCs, however, is comparable to non-PGE2 matured DCs (99). This means that until now, no DC maturation strategy has been described to manipulate *in vivo* DC-migration. The percentage of DCs that reach the draining lymph node in clinical DC vaccination protocols is about 4% (100). To increase the number of DCs entering the draining lymph node, different DC injection techniques have been used. Next to the subcutaneous injection, DCs have been injected intranodally (101, 102), intratumoral (103), intravenous and intralymphatically (104). Only few studies with low patient numbers compared the effect of different injection methods on clinical responses (104). Additionally, these techniques appear difficult to perform and success rates completely depend on the

experience of the injecting radiologist (105). Therefore, we would like to propose a new option to compensate for the poor DC-migratory capacity, the tertiary lymphoid structure. This technique makes use of the capacity of TLR-matured DCs to produce large amounts of different chemokines (94). By manipulating chemokine production, DCs are able to selectively recruit effector cells for interaction. This is illustrated by the capacity of TLR-matured DCs to produce NK cell-recruiting chemokines (106) and thereby recruit NK cells (17, 21, 71). DC-mediated chemokine production is also attractive for recruitment of other immune effector cells (107). For CTL recruitment both CXCL10 and CCL5 have been shown important (108-110). Interestingly, by manipulating the chemokine profile of DCs different T_H cells can be recruited, thereby controlling T cell responses. Like CTLs and NK cells, T_H1 cell migration also depends on CXCL10 and CCL5 secretion (111). We hypothesize that by recruitment of all these effector cells by DCs the same interactions that take place in lymph nodes can be induced extranodally, thereby compensating for the lack of migration by *ex vivo* matured DCs. Another possibility is intratumoral injection of DCs, recruiting effector cells directly into the tumor. One should however take into account that the immunosuppressive effect of the tumor micro-environment could have detrimental effects on effector cell induction by DCs.

Combination therapy

After reviewing the possibility of combination therapies for cancer by using NK-DC crosstalk, DC vaccination could also be combined with other therapies that target additional immune cells, induce tumor cell lysis or modulate the tumor micro-environment. A short overview of these strategies is presented here.

Adoptive T cell transfer

In terms of combining different immunotherapeutic strategies, one could argue that DC-vaccination should be accompanied by adoptive T cell transfer. The capacity of DCs to induce tumor-specific T cell responses depends on the presence and activation status of these T cells. In cancer patients, tumor-specific T cells are often anergic or subjected to

silencing. Additionally, it is possible that the tumor-specific T cell is not present in the patient's natural T cell repertoire. Therefore, the transfer of specific T cells or *de novo* pre-T cells could do both, increase the prevalence of specific T cells thereby expanding the natural repertoire and providing T cells that are not subjected to silencing. It has been shown in mice that the combination of DC vaccination and adoptive T cell transfer led to a more robust antitumor response than the use of each treatment individually (112, 113). *De novo* development of tumor-specific T cells would be the ultimate goal for DC vaccination strategies. To date, however, it is still impossible to produce these cells *ex vivo* (114). Though, with the use of pre-T cells the T cell repertoire can be expanded and hopefully supplemented with the missing tumor-specific T cells (115). It can be envisioned that with the latter strategy the prevalence of tumor-specific T cells will be much lower. The positive effect of the use of pre-T cells, however, is strengthening of the immune system with new specific T cells that can not only fight tumors, but also infectious agents. Moreover, with pre-T cells the risk of auto-reactivity is very low, because both negative and positive selection of these autologous T cells take place *in vivo*. This makes the use of pre-T cells also attractive for immune reconstitution after the immunosuppressive effect of chemotherapy or stem cell transplantation (116). Additionally, in auto-immune patients that receive high dose immune suppressive drugs, T cell reconstitution could prevent development of opportunistic infections. Results on the use of pre-T cells are still preliminary, however our mouse studies indicate that these cells have the capacity to migrate into the thymus of humanized mice (115).

Antibody therapy

To enhance immune responses, not only cellular responses can be enhanced but also humoral responses. DC vaccination induces both immune responses and often tumor-specific antibodies can be detected after DC vaccination (16). Therefore, antigen presentation in MHC class I and II by DCs is obligatory and this depends on the characteristics of the antigen used by *ex vivo* antigen loading. Not all antigens used in DC vaccination studies possess these characteristics and are therefore only expressed in MHC class II. However, humoral responses can be enhanced by simultaneous injection of tumor-specific antibodies thereby improving tumor cell elimination due to complement

activation and ADCC (117). Furthermore, antibodies can target antigens expressed by tumor surrounding tissue, thereby manipulating the immune suppressive environment, they can bind tolerogenic ligands on immune cells and they can prevent induction of tolerance (117). Clinical trials in which DC vaccination is combined with antibodies inhibiting development of Tregs show an increase in antitumor responses (118). Also the use of tumor-specific antigens enhances DCs to opsonize tumor cells and present tumor antigens (119). Next to these effects, antibodies can activate NK cells due to their ADCC function, thereby not only enhancing their cytotoxicity, but also their cytokine secretion, which promotes NK-DC crosstalk (42). One could hypothesize that DC vaccination, combined with adaptive NK cell transfer and antibody vaccination, would be superior at inducing antitumor responses.

Immunotherapy and conventional anti-cancer therapy

Current antitumor therapy is mainly based on surgery, chemotherapy and radiotherapy. All these strategies have been shown to have immunomodulatory effects. Many of these therapies curtail the immune response against tumor cells. These immunosuppressive side effects are induced either directly, by inhibiting or killing effector cells, or indirectly, by provoking anergy or immune paralysis (120). This is well illustrated with the combined resection of the primary tumor as well as the tumor draining lymph nodes. Though cancer cells could have metastasized into these lymph nodes, this is also the location where antitumor immune cells become activated and start proliferating. Therefore radical resection of these lymph nodes does not always improve overall survival in cancer patients (121). Additionally, chemotherapy and radiotherapy have direct negative effects on immune cells due to their direct cytotoxicity, thereby inducing lymphopenia (122, 123). Moreover, many chemotherapeutics are used as immunosuppressants for the treatment of severe autoimmune disease. This applies to steroids, methotrexate and cyclophosphamide, which have been demonstrated to bias T cell polarization toward a T_H2 response (124), inhibit DC maturation (125) and inhibit NK cell proliferation and cytotoxicity (126).

However, accumulating evidence indicates that innate and adaptive immune responses make a crucial contribution to the antitumor effects of conventional chemotherapy- and

radiotherapy-based cancer treatment (120, 127). Surgical resection of most of the tumor mass prevents the production of immunosuppressive factors by tumor cells, thereby reducing the suppressive effect on the immune system. Radiotherapy and chemotherapy have the same effect since they also induce apoptosis of tumor cells, thereby decreasing tumor burden. Additionally, they induce other immunomodulating mechanisms. One of these mechanisms is induced by the direct apoptotic effect of both therapies. By the induced cell death, antigenic material becomes available for DCs to take up and efficiently present to T cells (128, 129). Additionally, the toxic effect enhances expression of stress factors by tumor cells due to DNA-damage, which enhances expression of NKG2D ligands on tumor cells that ligate with the NKG2D (130). Also upregulation of other stress factors enhances NK cell-mediated tumor cell death. Unpublished work by Frings et al., indicates that murine breast cancer cells upregulate specific NK cell stimulating molecules after chemo- and radiotherapy, thereby enhancing the effect of haploidentical stem cell and NK cell transplantation (Frings et al. manuscript in preparation and (131)). Upregulation of stress factors has also been shown to amplify DC-mediated responses due to secretion of HMGB1 by apoptotic cells, which triggers TLR4 and thereby enhances DC activation and cross-presentation of tumor antigens (132). Low dose radiation is known to upregulate the expression of MHC class I molecules, tumor-associated antigens (including Mucin 1), the FAS death receptor and adhesion molecules (133, 134). Due to these immunostimulatory effects, radiation of a single tumor site can induce tumor regression of non-irradiated metastasis (134). Lymphoablation induced by low dose total body irradiation combined with chemotherapeutics does not only have detrimental effects on the immune system, but also opens opportunities for adoptive cell transfer. Due to the induction of transient lymphopenia, Tregs are eliminated as well and lymphoid reconstitution could overcome immune cell anergy (135, 136). Additionally, low dose cyclophosphamide has direct T and NK cell stimulatory effects, augmenting their proliferative and cytotoxic capacity (137). Both in mice and humans, several anti-cancer chemotherapeutic agents have been used in combination with immunotherapy and results are promising. The effect on NK cell cytotoxicity by DC vaccination strategies can be improved by pretreatment with 5-fluorouracil (34). Also the use of adjuvant oxaliplatin and capecitabine enhances the effect of DC vaccination (127, 138). Moreover, DC vaccination was able to increase

chemosensitivity of previously chemoresistant tumors (139). A combination of doxorubicin with IL-12 therapy prolonged survival of mice with leukemia (140). In extension with these findings, it can be anticipated that other immunomodulating drugs could boost anticancer immune responses. These drugs are not classified as chemotherapeutics, but have an effect on the tumor micro-environment. They include agents that inhibit tumor microvasculature like VEGF (141) and also cyclooxygenase (COX) inhibitors, which have been shown to inhibit tumor cell proliferation and migration but enhance apoptosis and immune cell invasion (142). Since COX2 inhibition prevents the formation of PGE2, it also prevents the inhibitory effect of PGE2 on immune cells (143). Previously, it has been shown that the combined use of DC vaccination and COX2 inhibitors is superior at inducing antitumor responses than DC vaccination alone (144, 145).

In conclusion, though conventional therapy, as well as immunotherapeutic strategies have been shown to induce antitumor responses, relapses are still frequent. To win the fight against cancer it is not only necessary to kill all cancer cells, but also chemoresistant cancer stem cells and induce protective immune responses. Therefore, combination strategies including both conventional and immunotherapy could enhance antitumor responses, break anergy and protect the patient from tumor recurrence (146).

Monitoring immune responses

New insights in interactions of different immune cells can be provided by closely monitoring immune responses. It has been demonstrated extremely difficult to identify a readout methods, that correlates best with clinical outcome. Different immunomonitoring strategies have been developed (Table III), they are, however, not applied in all immunotherapeutic trials.

Table III monitoring systems for cancer therapy

Responses	Methods	Reference
Clinical responses	Tumor size and metastasis (Radiology) Tumor markers (PSA, CEA)	(147, 148)
DCs	Migratory capacity (cell tracking) Cytokine production	(149)
NK cell	Phenotype (expression of NCRs and activating molecules) Cytokine production (IFN- γ , TNF- α) Cytotoxic capacity	(30)
Antibodies	Tumor-specific antibodies (monoclonal/polyclonal)	(150)
T helper induction	Signature cytokine production Signature gene expression	(151-153)
CTL cell responses	Presence of tumor-specific CTLs (or KLH/DTH responses) Cytotoxic capacity of tumor-specific CTLs	(154)

Future perspectives

For immunotherapy to be an effective therapeutic option, clinical trials should be performed. A major drawback of immunotherapy, compared to conventional anticancer therapy, is that it is a patient-specific therapy. This makes it very expensive and unattractive for most companies to invest in. Though over the past few years many cell therapy companies have been founded, only few major clinical trials have been performed. To reduce the costs of immunotherapy, many investigators have been studying the possibility of an off-the-shelf product. Examples are the use of immune cells developed from autologous or allogeneic stem cells that are genetically altered to specifically target antigens expressed by the majority of tumors. Also cell lines can be used for this purpose. However, I am convinced that the development of these products can only be effective if we have elucidated the different responses induced by regular immunotherapy. With these new therapies we have to overcome many more hurdles, including transplantation over HLA-borders. Therefore, I would suggest to perform clinical immunotherapeutic trials, where not only clinical responses are investigated, but also immune responses. Additionally, these responses should be analyzed in patients treated

with conventional anticancer therapy. We should learn more about the nature of immune responses induced to determine the possible additive effects.

Over the past years, vaccination strategies have been extensively studied *in vitro*. Different maturation cocktails have been used, different immune interactions have been studied and different tumor targets have been investigated. With increasing knowledge it is now possible to produce DCs with better *in vitro* capacities than previous DCs that were only scarcely used in clinical trials. Next to these developments, also genetically altered DCs that can be used as an off-the-shelf product are being developed. But where we seem to get stuck is the translation into clinical practice. This is nicely illustrated by the quest of developing DCs that are able to migrate *in vitro*. Many different groups have investigated the behavior of DCs matured by different factors that either enhance or inhibit DC migration. However, *in vivo* all DCs seem to migrate just as poor and development of *in vitro* migrating DCs seems of no *in vivo* or clinical use. Therefore, immunotherapy screams for clinical trials to be used in regular anticancer, since *in vitro* optimization is only useful if we know what the *in vivo* problems are.

In nearly every review on immunotherapy, one of the biggest problems in effectiveness of immunotherapy is the patient population used in trials. In nearly every trial, end-stage cancer patients are included. One could reason that the immune system in these patients suffers from the enormous suppressive effect of the tumor. Additionally, these patients were previously treated with different regimens of radio- and chemotherapy, thereby already selecting the most aggressive tumor cells. The use of immunotherapy as an adjuvant in current therapeutic strategies might be very appealing, since these adjuvants could possibly induce protective immune responses. Tumor immune surveillance would be enhanced, prohibiting tumor cells to grow. Hopefully, this will translate into increased tumor-free survival and decreased recurrence rates.

Figure 4. The Holy Grail of anticancer therapy.

With our present knowledge it is an euphemism to think that there is a single anticancer agent that serves as the Holy Grail in antitumor responses. Until we have found this strategy, we have to combine different therapeutic options that either activate the immune system, manipulate the tumor microenvironment and efficiently use the tumor characteristics to induce stable antitumor responses.

In my opinion, the key to development of effective antitumor protocols lies in performing clinical studies and acknowledging the fact that anticancer therapy is not a one man show. It depends on the tumor characteristics, the tumor microenvironment, activation and suppression of the immune system and of course on the patient's condition. History has proven that there is not one therapy that ticks all the boxes (Holy Grail) and therefore different antitumor strategies have to cooperate (Figure 4): cooperation of different immune cells and cooperation of conventional therapy with immunotherapy. Of course these combinations will not only augment anticancer responses, also inhibition is possible, therefore clinical effectiveness should be very closely monitored, with a special regard to immune responses.

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Chapter 9

Summary

Summary

In this thesis we studied various aspects of different immunotherapeutic therapies for cancer *in vitro*. The aim was to improve clinical outcome by combining different immunotherapeutic approaches. We thereby present methods for combination therapy and provide tools to evaluate immunologic responses.

In **chapter 2** we investigated the possibility to utilize passive antibody transfer for ovarian cancer. We show that most ovarian tumors and their metastasis express underglycosylated MUC1 (MUC1-Tn/STn), whereas healthy ovarian cells don't. Therefore, we reason that MUC1-Tn/STn epitopes could serve as tumor-specific antigens, which can be targeted with adoptive transfer of humanized antibodies or DC vaccination in immunotherapy for ovarian cancer.

To optimize DC-based vaccination strategies, we show in **chapter 3** that TLR2/4-matured DCs are superior in inducing T_H1 polarization and additionally induce more MUC1-specific CTLs as compared to PGE2-matured DCs. Additionally, in **chapter 4** we show that these TLR2/4-matured DCs are also superior in interaction with NK cells. This interaction does not only enhance NK cell-mediated cytotoxicity, but also IFN- γ -dependent T_H1 polarization. The use of PGE2 in DC maturation protocols is based on the positive effect of PGE2 on the DC's migratory capacity. Indeed, though TLR2/4-matured DCs are superior in activating immune effector cells, they display a very poor migratory capacity *in vitro*. By analyzing the chemokine profile of these TLR2/4-matured DCs, which produce high amounts of T_H1 , CTL and NK cell-recruiting chemokines we have formed the basis of a new approach for DC vaccination strategies. As opposed to previous reports on enhancement of DC migration, we reason that if *ex vivo* developed DCs are such poor migrators, they should be stimulated to recruit effector cells. For NK cells we show that TLR2/4-matured DCs do this very efficiently in a CCR5-dependent mechanism.

In terms of combination therapy, we investigated possible additional factors produced by tumors, which inhibit antitumor immune responses. One important factor is PGE2, a potent immune suppressor, which is secreted by many solid as well as hematological malignancies. Paradoxically, this is the same factor often used in DC maturation protocols. PGE2 has previously been shown to inhibit both innate as well as adaptive immune

responses. Interestingly, in **chapter 5** we show that PGE2 has also detrimental effects on NK-DC interaction. PGE2 directly affects NK cell function by inhibiting cytotoxicity and cytokine production and also indirectly via DCs it inhibits NK-DC crosstalk. PGE2-matured DCs are less capable of recruiting and activating NK cells, which we have shown in **chapter 4** to be essential for T_H1 polarization.

To evaluate the effect of DC vaccination, we state that different immune responses should be monitored. Next to the conventional T cell monitoring techniques, including DTH-reactions, tetramer staining and cytokine profiles also NK cell activation should be monitored. Additionally, we developed the tools to evaluate humoral immune responses. In **chapter 6** we describe a flow cytometry-based assay to detect human serum antibodies to MUC1 and MUC1-Tn/STn epitopes.

To enhance the effectiveness of cellular immune responses by DC vaccination, we investigated the possibility to develop *ex vivo* generated T cells that are not subjected to tolerance during development. In **chapter 7** we show that we are able to develop these cells that hold the capacity to develop into mature T cells in humanized mice models. Though our data are still preliminary, one could envision that these pre-T cells can be developed into tumor-specific T cells *in vivo* or *ex vivo* to be adoptively transferred.

In conclusion, in this thesis we present an anti-MUC1 strategy for anti-cancer therapy, which could be used in many different patients with either solid tumors or hematological malignancies that express MUC1 (Figure 1). Since we do not expect immunotherapy to be effective by targeting individual immune cells, we propose to combine adoptive antibody therapy with TLR2/4 matured DC vaccination, adoptive pre-T cell transfer and possibly COX2 inhibition to reduce the effect of tumor-derived PGE2 on immune responses. To evaluate immune responses innate as well as adaptive and humoral as well as cellular responses should be monitored.

Figure 1. Summary of this thesis: combination therapy

In terms of combination therapy, different immunotherapeutic strategies can be combined. (2) tumors expressing tumor-specific antigens can be targeted by adoptive antibody transfer. (3) DC vaccination can be utilized to induce tumor-specific T cells and (4) activation of NK cells, by recruiting these cells creating a tertiary lymphoid structure, and enhancing their cytotoxic as well as their cytokine secreting capacity. (5) Tumor-derived PGE2, which has immunosuppressive characteristics should be inhibited by COX2- inhibitors. (7) Additionally, to replenish the immune effector population effector cells, like T cells, can be adoptively transferred. (6) Finally, to evaluate immune responses innate as well as adaptive and humoral as well as cellular responses should be monitored. The different numbers also correspond to the different chapters in which the issues were addressed.

Samenvatting

Nederlandse samenvatting

In dit proefschrift hebben we verschillende *in vitro* aspecten van immunotherapie voor kanker bestudeerd met als doel de klinische resultaten te verbeteren door verschillende immunotherapeutische strategieën te combineren. We beschrijven nieuwe methodes die als combinatietherapie gebruikt kunnen worden voor kanker patiënten. Daarnaast presenteren we een innovatieve manier om immuunresponsen te analyseren.

In **hoofdstuk 2** hebben we onderzocht of antilichaam therapie effectief kan zijn voor patienten met ovarium tumoren. Eén van de vereisten hiervoor is de expressie van tumorgeassocieerde antilichamen door de tumorcellen. In deze context hebben we aangetoond dat de meerderheid van de ovariumtumorcellen ondergeglycosyleerd MUC1 (MUC1-Tn/STn) tot expressie brengen. Niet alleen de primaire tumoren zijn hiermee mogelijk gevoelig voor antilichaamtherapie, maar ook de metastasen brengen dit ondergeglycosyleerde MUC1 tot expressie, terwijl gezonde ovarium cellen dit niet doen. Hieruit concluderen we dat de MUC1-Tn/STn epitopen gebruikt kunnen worden als tumorspecifieke antigenen in ovarium kanker en dat deze antigenen getarget kunnen worden met antilichamen of middels DC vaccinatie strategieën.

Ter optimalisatie van DC vaccinatie strategieën laten we in **hoofdstuk 3** zien dat TLR2/4 gematureerde DCs superieur zijn aan PGE2 gematureerde DCs. TLR2/4 gematureerde DCs induceren voornamelijk T_H1 polarisatie en induceren meer antigeenspecifieke CTLs. Daarnaast laten we in **hoofdstuk 4** zien dat deze TLR2/4 gematureerde DCs ook veel beter zijn in de communicatie met NK cellen. Hierbij ontstaat er een bidirectionele activatie van DCs en NK cellen, waarbij NK cellen gestimuleerd worden tot het induceren van celgemedieerde cytotoxiciteit en inductie van $IFN-\gamma$ afhankelijke T_H1 polarisatie. PGE2 wordt frequent gebruikt in DC maturatie protocollen, gezien het positieve effect op de *in vitro* migratie van DCs. In vergelijking met PGE2 gematureerde DCs vertonen TLR2/4 gematureerde DCs inderdaad een vele slechtere capaciteit tot migreren. Echter, bij analyse van het chemokine profiel van deze TLR2/4 gematureerde DCs blijkt dat deze grote hoeveelheden aan T_H1 , CTL en NK celrekruterende chemokines produceren. Deze data leggen de basis voor een nieuwe strategie voor DC vaccinatie, waarbij het migrerend vermogen van de DC niet meer centraal staat, maar juist het rekruterend vermogen veel

belangrijker is. Voor TLR2/4 gematureerde DCs laten we zien dat ze NK cellen rekruteren middels eens CCR5 gemedieerd mechanisme.

In relatie tot combinatie therapie hebben we additionele factoren onderzocht die geproduceerd worden door tumoren en die een negatieve invloed hebben op de immuunrespons. Gezien de negatieve effecten van PGE2 op DC maturatie en gezien veel solide tumoren evenals hematologische maligniteiten hoge concentraties PGE2 produceren hebben we het effect van PGE2 op NK-DC interactie verder bestudeerd. Van PGE2 is bewezen dat het zowel de aangeboren als de verworven immuniteit negatief kan beïnvloeden. In **hoofdstuk 5** laten we zien dat PGE2 een negatief effect heeft op NK-DC interactie. PGE2 heeft een direct effect op NK cellen, waarbij de cytokine secretie en cytotoxiciteit van NK cellen gehinibeerd wordt en daarnaast inhibeert PGE2 ook NK-DC interactie door de negatieve invloed van PGE2 op DC maturatie.

Om te evalueren of DC vaccinatie succesvol is, is het van belang dat naast de klinische responsen ook de immunologische responsen geëvalueerd worden. Naast de conventionele methoden waarbij T cel activatie geanalyseerd wordt, middels DTH-reacties, tetrameer analyse en cytokine profiel, moeten ook NK cell responsen worden geëvalueerd. Daarnaast hebben we een nieuwe methode ontwikkeld om humorale immuunresponsen te analyseren. In hoofdstuk 6 beschrijven we deze op flow cytometrie gebaseerd methode, waarbij humane serum antilichamen tegen MUC1 en MUC1-Tn/STn epitopen gedetecteerd kunnen worden.

Om de effectiviteit van de cellulaire immuunresponsen die door DC vaccinatie geïnduceerd worden te verbeteren hebben we gepoogd om T cellen *ex vivo* te genereren uit stamcellen. Hiermee kunnen T cellen gemaakt kunnen worden die tijdens hun ontwikkeling niet onder druk van immuunsuppressie staan. In **hoofdstuk 7** laten we zien dat we *ex vivo* pre-T cellen kunnen ontwikkelen die in staat zijn uit te groeien tot mature T cellen in een gehumaniseerd muis model. Onze data zijn nog steeds preliminair, maar mogelijk kan met deze methode uit *ex vivo* gegenereerde pre-T cellen *in vivo* tumorspecifieke T cellen ontwikkeld worden. Dit zou een potentiële therapieoptie zijn, die in combinatie met een DC vaccine toegediend kan worden.

In dit promotieonderzoek presenteren we een anti-MUC1 strategie die toegepast kan worden in patiënten met solide tumoren evenals in patiënten met hematologische

maligniteiten (Figuur 2). Gezien we ervan overtuigd zijn dat immunotherapie alleen succesvol zal zijn indien meerdere strategieën gecombineerd worden en daarmee ook verschillende immuuncellen getarget worden, stellen we voor om antilichaam therapie te combineren met DC vaccinatie, pre-T cell toediening en COX2-inhibitie om het effect van PGE2 te onderdrukken. Om het effect van deze therapie te evalueren dienen naast klinische responsen ook de immunologische responsen geëvalueerd te worden.

Figuur 2. Samenvatting van dit proefschrift: combinatie therapie

Voor combinatietherapie kunnen verschillende immunotherapeutische strategieën gecombineerd worden. (2) Tumoren brengen tumorspecifieke antigenen tot expressie, die getarget kunnen worden middels antilichaam therapie. (3) DC vaccinatie kan gebruikt worden om tumorspecifieke T cellen te induceren en om (4) activatie van NK cellen te bewerkstelligen. Hiervoor zullen beide cellen door de DC gerekruteerd moeten worden, waarbij een tertiaire lymfoïde structuur gevormd wordt. (5) PGE2 dat geproduceerd wordt door veel tumoren, heeft een immuunsuppressieve invloed, hetgeen middels COX2-inhibitoren opgeheven kan worden. (7) Om het T cel repertoire aan te vullen, kunnen deze effectorcellen *ex vivo* worden gegenereerd om samen met een DC vaccine toegediend te worden. (6) Als laatste dienen cellulaire evenals humorale immuunresponsen geëvalueerd te worden. De verschillende nummers corresponderen met de hoofdstukken waarin de onderwerpen besproken worden.

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Softbal is een teamsport en daarbij zijn je teamgenoten erg belangrijk. Goede teamgenoten heb ik dan ook gevonden in collegae promovendi, postdocs en analisten. Bij softbal heb je mensen nodig die goed kunnen slaan, zodat de mensen die goed kunnen rennen de punten kunnen maken. Ook in het veld heb je altijd een man achter je die de bal kan vangen, indien hij uit de handen glipt. Zo is het ook tijdens mijn promotie geweest, waarbij iedereen zijn eigen speciale plaats heeft gehad. Birgit, jij was de beste pitcher van ze allemaal. Niet alleen op het lab, maar ook tijdens een echt spelletje softbal heb ik gemerkt met hoeveel fanatisme jij je erop stort (zelfs kleine meisjes moeten het ontgelden als jij de overwinning ruikt). Je was er altijd met zowel praktische als met persoonlijke adviezen. Als ik het tegenwoordig erg druk heb en te veel dingen tegelijk moet doen, dan mis ik ons Balisto-momentje nog steeds.

Het eerste honk is een hele belangrijke plaats, het is het eerste punt waar de tegenstander tegengehouden kan worden. Bob, je hebben op deze plaats als eerste verdediging prima gefunctioneerd. Door vaak in een vroeg stadium al met jou te overleggen, hoefde ik het wiel niet opnieuw uit te vinden en is me veel extra werk bespaard gebleven. Bob, helaas hadden we niet voldoende sponsoring om je in het team te houden en ben je door een groter en professioneler team weggekaapt. Alle discussies met jou waren van zeer hoge wetenschappelijke kwaliteit, jij zorgde ervoor dat ik uitgedaagd werd en liet me zien dat wetenschap een fantastische hobby is. Ook al moesten we eerst aan elkaars karakter wennen, uiteindelijk is er een fantastische vriendschap uit ontstaan en heb ik nog nooit zo veel chocolade taarten voor iemand gebakken.

Peter, het was geweldig om met jou samen op het tweede honk te staan (ook al zijn we persoonlijk nooit tot second base of zelfs first base gekomen). Je kwam er als eerste achter hoe geschrift ik eigenlijk was. Zo heb je je meermaals moeten schamen met mij in de file en kan ik me voorstellen dat je nooit meer iemand in de auto hebt horen roepen: smiley, smiley, smiley! Congressen waren een feestje. Gelukkig waren we samen, anders

hadden we vast wel ergens een keer een vlucht gemist op weg naar Australië. Ook heb ik nog nooit iemand zo blauw met z'n snorkel uit de zee zien komen. Kortom we hebben veel lol met elkaar kunnen delen en als roommates hebben we veel onzin uitgehaald, zeker tijdens het EK van 2008 toen er een geur van rubberen ballonnen uit onze kamer kwam, aangevuld met een oranje gloed.

Dan het derde honk, waar meerdere spelers hebben gestaan, die uiteindelijk ook door andere clubs zijn weggekaapt. Silvie, in het Pacrima project was je altijd duidelijk bezig met de klinische implicatie. Daarnaast heb je ons door Tongeren geleid en zal ik nooit meer vergeten wat een vidéke is. Ariane, zelfs vlak voor mijn promotie heb je me nog geholpen met informatie. Jullie twee zijn al die jaren mijn voorbeeld geweest en tijdens jullie promotie was in zenuwachtiger dan jullie. En natuurlijk Mariska, met jou op het NVVI op een kamer staat garand voor een nacht zonder veel slaap. Ik ben blij dat jullie alle drie op jullie eigen manier het geluk hebben gevonden in jullie leven.

Natuurlijk moeten de we catcher niet vergeten. Lotte, gelukkig kwam jij via een transfer vanuit Utrecht naar het zuiden. Niet alleen de mannelijke teamgenoten waren blij met de aanschaf van deze blonde sterspeelster, maar ook ik was blij dat je bij het team kwam. Eindelijk was er een vrouwelijk iemand die net zo veel lol in wetenschap had als ik. Iemand die ook alles voor haar onderzoek over heeft en daarnaast heb ik nog nooit zo in een deuk gelegen tijdens experimenten dan met jou op het muizenlab in Utrecht.

Dan is natuurlijk ook het buitenveld van belang, Ans, Catharina, Melanie en ook de nieuwe lichting promovendi Subash, Tammie en Mirelle hebben wetenschappelijk hulp geboden en voor veel vermaak gezorgd. Velen van jullie zijn vanuit het buitenveld al in het binnenveld gekomen. Vooral jou Tammie wil ik bedanken voor je hulp en ik wens je veel succes met het vervolg van het NK-DC-T cel werk.

Natuurlijk heeft elk team ook een sponsor nodig. Hierbij heb ik het geluk gehad een hele speciale sponsor te hebben, die gewoon mijn werkgever was "PharmaCell". Dank je wel Rene om mij aan te nemen als junior scientist en bedankt Maurice Horsten en Alexander Vos, dat jullie ook in moeilijker tijden mijn sponsor zijn gebleven. Ook al lagen de prioriteiten vaak ergens anders, jullie hebben altijd je best gedaan om mij bij het bedrijf te betrekken, zelfs bij elk bedrijfsfeestje werd ik uitgenodigd. Een speciaal bedankje hierbij ook voor Marianne. Als er een prijs was voor beste secretaresse ooit, zou jij hem winnen.

Daarnaast wil ik ook wijlen Jan Thio niet vergeten als initiatiefnemer van het Pacrimaproject. Het project dat de succesvolle samenwerking tussen de Universiteit en Pharmacell tot stand heeft gebracht.

Dan een grote dank aan het publiek. Vicky, je was niet alleen een collega, maar je bent ook een vriendin geworden en ik ben heel blij dat jouw grootste droom afgelopen jaar uit is gekomen. Marcel en Nordin, jullie zijn alle twee op jullie eigen manier compleet geschild en ook al willen jullie vaak een beetje dood, ik weet zeker dat jullie boekje fantastisch gaat worden. Ook dank je wel aan de mede commissie leden van ProvUM, speciaal Cynthia, Floris, Mirjam en Jennifer met de belangen van de promovendus op de eerste plaats hebben toch ook heel veel lol gemaakt.

Tja en bij het publiek horen natuurlijk ook de mensen die al die jaren op de eerste rij zitten en me aanmoedigen. Pap, Mam met veel trots, maar vaak ook gemengde gevoelens hebben jullie toegekeken hoe ik soms punten scoorde en soms ook flink getackeld werd. Jullie waren er altijd voor me, ook als ik heel laat op de avond of nacht nog op een incubatie moest wachten, werd bij jullie altijd de telefoon opgenomen. Als ik laat het 's avonds naar huis ging en langs jullie huis reed, brandde er altijd nog wel een lampje. Nog steeds staan jullie altijd voor ons klaar. Dank je wel, is eigenlijk te weinig, maar ik geloof dat jullie dat wel begrijpen.

En ook al wil je het zelf niet, ook jou Daniël wil ik bedanken. Ik denk dat geen enkele vriend mijn gedrag van de afgelopen jaren zou hebben geaccepteerd. Ik was meer op mijn werk dan thuis, maar dat deed jou helemaal niets. Mijn werk, mijn promotie ging voor alles en daar zette jij je ook volledig voor in. Als ik in het weekeinde weer moest werken, ging je gewoon mee (misschien had de snoepautomaat daar ook iets mee te maken). Als ik thuis zat te werken kwam je me drinken brengen en als ik de studeerkamer weer eens ging verbouwen uit frustratie, dan liet je me rustig mijn gang gaan totdat ik weer bedaard was. Jou heeft het letterlijk bloed (veel bloed), zweet en tranen gekost.

Tja en dan heb je nog de tegenstander. Daar zijn de reviewers, de beoordelaars van projecten en misschien wel de grootste tegenstander mijn eigen ambitie. Vooral het laatste is een ziekte gebleken, die helaas besmettelijk is (hè Daniël?). Toch wil ik al deze tegenstanders bedanken, want zonder jullie commentaar was ik nooit zo ver gekomen.

Uiteindelijk kan ik zeggen dat ik in de afgelopen jaren een interessante wedstrijd gespeeld heb, waarbij we innings hebben gewonnen en verloren. Ik heb de stand niet bijgehouden en ben ik er ook nog niet zo zeker wie gewonnen heeft, maar uiteindelijk kan ik zeggen: It was one hell of a game.

List of publications

List of publications

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Curriculum vitae

Curriculum Vitae

Catharina, Hubertina, Maria, Johanna Van Elssen werd geboren op 2 november 1982 te Heerlen. Na het behalen van haar VWO diploma in 2000 aan het Grotius College te Heerlen werd gestart met de studie geneeskunde aan de Universiteit van Maastricht. Tijdens haar studie participeerde ze in het onderzoek naar methylering van oncogenen in renaal cel tumoren bij de afdeling pathologie van het Academisch ziekenhuis Maastricht. Daarnaast deed ze onderzoek naar de expressie van ondergeglycosyleerd MUC1 bij ovarium tumoren bij de afdeling tumor immunologie eveneens in Maastricht. In 2006 werd het artsexamen behaald (cum Laude) en startte ze als PhD student bij de afdeling tumorimmunologie, later hematologie, aan haar promotietraject aangaande immunotherapie voor kanker onder de supervisie van Dr. G.M.J. Bos en Prof. Dr. H.C. Schouten. Dit onderzoeksproject kwam voort uit een samenwerking tussen het Universitair Medisch centrum Maastricht en het celtherapie bedrijf Pharmacell, hetgeen resulteerde in een dubbele aanstelling. Tijdens haar promotieonderzoek nam ze plaats in het bestuur van de promovendi vereniging ProVUM van de Universiteit Maastricht waar ze van 2008 tot 2010 penningmeester was.

Vanaf september 2010 is zij werkzaam als assistent in opleiding tot internist in het Universitair Medisch Centrum Maastricht (MUMC) met als opleider Prof. Dr. C.D.A Stehouwer en plaatsvervangend opleider Prof. Dr. R.P. Koopmans.