



Lange Kleiweg 137
P.O. Box 45
2280 AA Rijswijk
The Netherlands

TNO report

DV2 2005-A29

Generic therapy after exposure to biological
warfare agents: Validation of an in vitro dendritic
cell culture system

www.tno.nl

T +31 15 28 43000
F +31 15 28 43991
Info-DenV@pml.tno.nl

Date	May 2005
Author(s)	Dr. D. van der Kleij Dr. M. Polhuijs
Classification report	Ongerubriceerd
Classified by	Dhr. A.S. de Koning
Classification date	25 April 2005 (This classification will not change)
Title	Ongerubriceerd
Managementuittreksel	Ongerubriceerd
Abstract	Ongerubriceerd
Report text	Ongerubriceerd
Copy no	21
No. of copies	22
Number of pages	24 (excl RDP and distribution list)
Number of appendices	0

DISTRIBUTION STATEMENT A
Approved for Public Release
Distribution Unlimited

The classification designation Ongerubriceerd is equivalent to Unclassified.

All rights reserved. No part of this report may be reproduced in any form by print, photoprint, microfilm or any other means without the previous written permission from TNO.

All information which is classified according to Dutch regulations shall be treated by the recipient in the same way as classified information of corresponding value in his own country. No part of this information will be disclosed to any third party.

In case this report was drafted on instructions from the Ministry of Defence the rights and obligations of the principal and TNO are subject to the standard conditions for research and development instructions, established by the Ministry of Defence and TNO, if these conditions are declared applicable, or the relevant agreement concluded between the contracting parties.

© 2005 TNO

AQ F06-11-7958

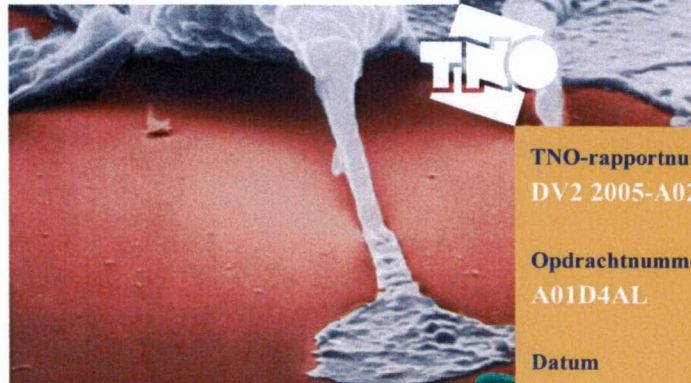
Generieke therapie B-agentia: validatie van een kweekstelsel van dendritische cellen

Probleemstelling

De ontwikkelingen na de Golf-oorlog alsmede de onthullingen over het Biopreparaat conglomeraat in de voormalige Sovjet-Unie geven duidelijk aan dat de dreiging van het gebruik van biologische wapens, waaronder bacteriën, is toegenomen. Bacteriële infecties veroorzaakt door het gebruik van biologische strijdmiddelen, zijn veelal te bestrijden met antibiotica. Door spontane (*Yersinia pestis* in Madagascar) of opzettelijke (Biopreparaat conglomeraat in de voormalige Sovjet-Unie) mutaties kan het voorkomen dat een bacteriestam resistent is geworden voor het antibioticum van eerste keus. Daarnaast levert het gebruik van genetische manipulatie de mogelijkheid om op eenvoudige wijze een grote verscheidenheid aan biologische wapens te produceren. Het is onmogelijk om tegen al deze producten de afzonderlijke vaccins te ontwikkelen en, gezien het ontstaan van een mogelijk Golf-oorlog Syndroom, deze vaccins aan de militair toe te dienen. Een meer generieke aanpak waarmee de effecten van een groot scala aan bacteriën worden voorkomen, lijkt een effectievere aanpak.

Bij kankerpatienten wordt reeds getracht het afweersysteem specifiek te stimuleren door middel van het toedienen van immunomodulators. Immunomodulators zouden mogelijk ook kunnen worden toegepast om het afweersysteem van militairen op niet-specifieke wijze te stimuleren na blootstelling aan een breed scala aan bacteriën.

In opdracht van het Ministerie van Defensie wordt de haalbaarheid van deze aanpak onderzocht door TNO. Om de effecten van biologische agentia op het immuunsysteem in kaart te brengen en therapieën te kunnen screenen, werd een *in vitro* kweekstelsel opgezet. Dit onderzoek is uitgevoerd door



TNO Defensie en Veiligheid, locatie Rijswijk. Het onderzoek is gefinancierd in het kader van het programma 'Passieve verdediging tegen NBC-wapens' (V013) en beantwoordt aan resultaatnummer 808.

Beschrijving van de werkzaamheden

Allereerst werd in de literatuur nagegaan welk *in vitro* modelstelsel gebruikt kan worden om de effecten van militair relevante biologische agentia op het immuunsysteem van de mens te testen. Aangezien dendritische cellen een centrale rol spelen in de opbouw van een immunorespons tegen ziekteverwekkers, werd gekozen om te werken met dendritische cellen van de mens. De beschikbare hoeveelheid van dit type cellen in bloed is echter niet voldoende groot. Daarom werd gekozen om met behulp van groeifactoren monocyt (precursorcellen voor dendritische cellen) uit bloed te laten ontwikkelen tot dendritische cellen. Dit modelstelsel werd gevalideerd door modelstoffen toe te voegen waarvan het *in vivo* effect op ontwikkeling van dendritische cellen bekend is (LPS, IFN- γ , PGE₂). De effecten van deze componenten op dendritische cellen werden geanalyseerd door de aanwezigheid van moleculen op het celoppervlak en productie van signaalmoleculen te meten.

TNO-rapportnummer
DV2 2005-A029

Opdrachtnummer
A01D4AL

Datum
mei 2005

Auteur(s)
dr. D. van der Kleij
dr. M. Polhuijs

Rubricering rapport
Ongerubriceerd

Resultaten en conclusies

Het in vitro kweekstelsel van dendritische cellen is opgezet en de respons van het stelsel op modelstoffen komt overeen met de verwachting.

Toepasbaarheid

Indien militairen zijn blootgesteld aan een B-wapen, is er behoefte aan een snelle en efficiënte therapie. Indien de identiteit van het agens alsmede kennis over een eventuele antibioticumresistentie niet bekend zijn, zou een meer generieke therapie met immunomodulators uitkomst kunnen bieden. Door het niet-specifiek stimuleren van het afweer-

stelsel door deze therapieën zouden de effecten van blootstelling aan een groot scala aan biologische agentia (bacteriën) mogelijk kunnen worden geminimaliseerd. Aangezien een te sterke stimulatie van het immuunstelsel zou kunnen leiden tot ongecontroleerde ontstekingsreacties (sepsis), is inzicht in de regulatie van dergelijke reacties van groot belang.

De resultaten van het hierboven genoemde onderzoek laten zien dat het kweekstelsel van dendritische cellen een geschikt model is om de ontwikkeling van de immunorespons te bestuderen.

TNO

Lange Kleiweg 137
2288 GJ Rijswijk
Postbus 45
2280 AA Rijswijk

www.tno.nl
www.pml.tno.nl

T 015 284 30 00
F 015 284 39 91
E info@pml.tno.nl

PROGRAMMA	PROJECT
Programmabegeleider Dhr. A.S. de Koning, MGFB	Projectbegeleider Dr. H.J. Jansen, MGFB
Programmaleider Dr. ir. M.S. Nieuwenhuizen, Business Unit Biologische en Chemische Bescherming	Projectleider Dr. D. van der Kleij, Business Unit Biologische en Chemische Bescherming
Programmatitel Passieve verdediging tegen NBC-wapens	Projecttitel Generieke therapie B-agentia
Programmanummer V013	Projectnummer 014.12849
Programmaplanning Start 2001 Gereed 2005	Projectplanning Start 2001 Gereed 2005
Toezichthouder n.v.t.	
Frequentie van overleg Met de programma/projectbegeleider werd regelmatig gesproken over de invulling en de voortgang van het onderzoek.	Projectteam Dr. D. van der Kleij, dr. M. Polhuijs, ing. A.M. de Vries, Business Unit Biologische en Chemische Bescherming

Summary

Bacterial infections, amongst them those resulting from exposure to biological warfare agents, can be treated with antibiotics. However, because of the wide-spread use of these compounds, resistance of microorganisms against those antibiotics is becoming a large problem in the clinical setting. As a result, it might be possible that a bacterial infection cannot be treated efficiently with the antibiotic of choice and that precious time will be lost before a second, more efficient antibiotic compound can be administered.

In order to overcome these problems, vaccination of military personnel might be an option. However, it is not possible to develop vaccines against all possible threat agents. And even if this were feasible, it would not be wise to administer these vaccines to military personnel with the development of a possible Gulf War Syndrome in mind. A more generic approach to prevent the effects of a broad spectrum of bacteria seems more effective. In patients with cancer, the immune system is aspecifically stimulated with immunomodulators in addition to the treatment. It might be possible to use these compounds for aspecific stimulation of the immune system after an exposure to biological warfare agents.

To be able to study the development of immune responses against pathogens of military relevance to select potential broad-spectrum therapeutics, an *in vitro* culture system of dendritic cells was chosen. Dendritic cells play a central role in the coordination of developing immune responses. These cells are present in peripheral tissues of the body as sentinels to detect invading pathogens. Upon pathogen encounter, dendritic cells undergo a maturation process and migrate towards the lymph nodes, where they orchestrate the development of immunity via T helper cells.

A dendritic cell culture system was set up and was validated using model compounds of which the effects on dendritic cells are known. Bacterial lipopolysaccharide was used as a compound that induces maturation of dendritic cells, while interferon- γ and prostaglandin E2 were used to induce immune polarization towards type 1 and type 2 responses, respectively. The system was analyzed by measuring expression of surface markers on dendritic cells as well as cytokine production by dendritic cells and polarized T cells. All responses were in agreement with published literature in peer-reviewed journals.

Contents

	Managementuitreksel.....	2
	Summary	4
1	Introduction	6
1.1	Protection against biological warfare agents: a generic approach	6
1.2	Innate immunity and pathogens.....	6
1.3	The central role of dendritic cells in immune responses.....	7
1.4	Dendritic cells as an <i>in vitro</i> model for development of immune responses	8
2	Material and methods	9
2.1	In vitro generation of immature DC from monocytes	9
2.2	Induction of maturation of iDC	9
2.3	Analysis of expression of cell surface markers	9
2.4	Analysis of cytokine production by DC	9
2.5	Determination of naïve Thelper cell polarization by DC	10
2.6	Statistical analysis	10
3	Results.....	11
3.1	Isolation of monocytes from PBMC.....	11
3.2	Generation of immature DC	12
3.3	Maturation of DC	13
3.4	IL-12p70 production by DC	15
3.5	Polarization of T helper cell development by DC	15
4	Discussion and conclusions	17
4.1	Protection against biological warfare agents: a generic approach	17
4.2	The central role of dendritic cells in immune responses.....	17
4.3	Dendritic cells as an <i>in vitro</i> model for development of immune responses	18
4.4	Validation of the system	19
4.5	Future prospects.....	21
4.6	Points of consideration	21
5	References	23
6	Signature	24

1 Introduction

1.1 Protection against biological warfare agents: a generic approach

Bacterial infections, amongst them those resulting from exposure to biological warfare agents, can be treated with antibiotics. However, because of the widespread use of these compounds, resistance of microorganisms against those antibiotics is becoming a large problem in the clinical setting. As a result, it might be possible that a bacterial infection cannot be treated efficiently with the antibiotic of choice and that precious time will be lost before a second, more efficient antibiotic compound can be administered.

Resistant *Yersinia pestis* strains, most likely due to spontaneous mutation, were discovered in Madagascar. In addition, it is assumed that within the former Biopreparat conglomerat of the former Sovjet-Union, certain bacterial strains were genetically modified in order to become resistant against most of the commonly used antibiotics and vaccines. In literature, methods for the general approach of modification are well documented. As a result, the use of antibiotic resistant bacteria strains becomes a real threat.

In order to overcome these problems, vaccination of military personnel might be an option. However, it is not possible to develop vaccines against all possible threat agents. And even if this were feasible, it would not be wise to administer these vaccines to military personnel with the development of a possible Gulf War Syndrome in mind. But still, therapy is needed when military personnel is exposed to a biological warfare agent. A more generic approach to prevent the effects of a broad spectrum of bacteria seems more effective.

In patients with cancer, the immune system is aspecifically stimulated with immunomodulators in addition to the treatment. Immunomodulators from various categories are currently in use. Examples are microbial products, compounds of natural origin (calf thymic hormones, glucans, plant fractions), synthetic compounds (oligodeoxynucleotides containing CpG motifs, muramyl peptides, isoprinosine, imiquimod, linomide, pidotimod, LPS derivatives) and endogenous compounds such as cytokines [Masihi, 2000 and 2001]. It might be possible to use these compounds for aspecific stimulation of the immune system after an exposure to biological warfare agents.

1.2 Innate immunity and pathogens

Innate immunity is of great importance in the early phase of host defense. Innate immunity is mainly mediated by macrophages and dendritic cells (DC). These cells recognize conserved molecular patterns that are present in invading microorganisms.

A battery of surface molecules, among which the Toll-like receptor (TLR) family, serves as the pattern recognition receptors [Akira et al, 2001]. For instance, TLR-2 is activated by peptidoglycan of gram-positive bacteria whereas TLR-4 is activated by lipopolysaccharide (endotoxin) in the outer membrane of gram-negative bacteria.

The combination of pattern recognition receptors that is activated by a pathogen will determine the following effector response that is appropriate for dealing with the pathogen. However, pathogens have evolved numerous strategies to manipulate the immune system of their host to enhance survival of the pathogen. These strategies include suppression of inflammatory cytokines such as interleukin (IL)-12 and Tumor Necrosis Factor (TNF)- α , induction of anti-inflammatory cytokines such as IL-10, inhibition of DC maturation and downmodulation of expression of surface molecules on DC that are needed for induction of immunity. Immunomodulators may be used to counteract these evasion strategies of pathogens.

1.3 The central role of dendritic cells in immune responses

Dendritic cells are central players in the formation of immune responses (illustrated in Figure 1). DC are present in an immature state in peripheral tissues as sentinels to detect pathogens immediately upon invasion. The DC is equipped with a whole range of

so-called pattern recognition receptors (PRRs) which mediate recognition of microbial molecules. Upon activation through PRRs, DC start migrating towards the regional lymph node. During this migration, the DC matures and upregulates the expression of surface molecules that are essential for T cell activation, such as CD80, CD86 and HLA-DR. Within the lymph node, the DC meets T cells, and will activate T cells that have a T cell receptor that specifically recognizes peptides derived from the invading pathogen, resulting in pathogen-specific acquired immunity. The levels of cytokines produced by the DC during contact with T cells in combination with the surface molecules on the DC determine the type of acquired immune response that develops.

In case of an infection with intracellular bacteria a Thelper 1 type response will develop, characterized by T cells that produce IFN- γ , while an infection with extracellular pathogens such as parasitic worms will lead to development of a Thelper 2 type response, which is characterized by IL-4 producing T cells.

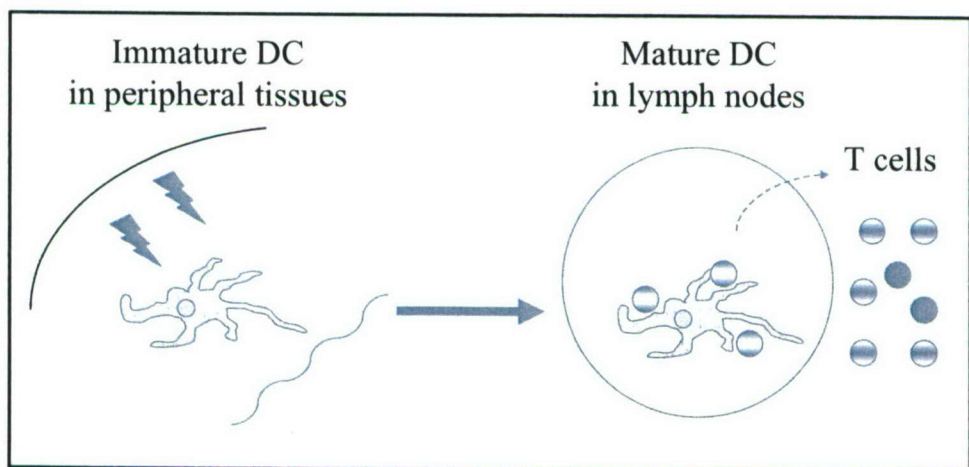


Figure 1 DC acquire information about invading pathogens in the periphery through innate pattern recognition receptors and subsequently initiate and regulate development of acquired immunity by T cell development.

1.4 Dendritic cells as an *in vitro* model for development of immune responses

In order to determine what type of immunomodulator will be beneficial to host immunity upon infection with pathogens, the effects of several threat agents on the host immune system must be determined first. Thereafter, appropriate immunomodulators can be selected, and their effects studied. The central role of the DC in the immune response makes this cell type highly suitable to study immune responses *in vitro*.

Therefore, an *in vitro* test system will be developed first to screen the effects of available immunomodulators. Thereafter, *in vivo* tests can be performed with compounds that showed promising results in the *in vitro* model. This report deals with the development of the *in vitro* test system.

2 Material and methods

2.1 *In vitro* generation of immature DC from monocytes

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats (Sanquin bloodbank Rotterdam) within 24 hours after venipuncture by density centrifugation on Ficoll Paque Plus (Amersham Pharmacia). To isolate monocytes, PBMC were centrifuged (1750g) for 45 min on a Percoll gradient consisting of three density layers (1.076, 1.059 en 1.045 g/ml). The light density fraction floating on the middle layer, containing primarily monocytes, was collected, washed and seeded in 24-well culture plates (Costar) at a density of 5×10^5 cells/well in RPMI 1640 (Biowhittaker) supplemented with 1% FCS (PAA). After 60 min at 37°C/6% CO₂, non-adherent cells were removed and adherent cells were cultured in RPMI supplemented with 10% FCS, 500u/ml recombinant GM-CSF (a kind gift from Joost Schuitemaker, AMC, Amsterdam) en 250u/ml recombinant IL-4 (Strathmann Biotech). At day 3, the culture medium including the supplements was refreshed. At day 6, CD1a⁺CD14⁻ immature DC were ready for use.

2.2 Induction of maturation of iDC

At day 6 or day 7, maturation of iDC was induced by the addition of 100ng/ml LPS from *E.coli* 055:B5 (Sigma). For polarization studies, recombinant IFN- γ (1000u/ml, Strathmann Biotech) was added in combination with LPS to induce Th1-promoting DC, while PGE2 (Sigma, 10 μ M) or Soluble Egg Antigens (SEA, 25 μ g/ml) from the parasitic worm *Schistosoma mansoni* (a kind gift from Dr. Maria Yazdanbakhsh, Leiden University Medical Center) was used in combination with LPS to generate Th2-promoting DC.

2.3 Analysis of expression of cell surface markers

Expression of cell surface markers was analyzed using mouse anti-human monoclonal antibodies against CD14-PerCP or CD14-FITC (BD Biosciences), CD45-PerCP (BD Biosciences), CD4-PE (BD Biosciences), CD8-PE (BD Biosciences), CD1a-PE (Immunotech), CD83-PE (Immunotech), HLA-DR-FITC (BD Biosciences) and CD86 (BD Biosciences), and was analyzed using a FACScan (BD Biosciences).

2.4 Analysis of cytokine production by DC

Two days after maturation, DC (2×10^4 cells/well) were stimulated with human CD40L-expressing mouse fibroblasts (2×10^4 cells/well) (J558 cells: a kind gift from Dr. P. Lane, University of Birmingham) in a 96 well flat-bottom plate (Costar) in RPMI 1640 containing 10% FCS in a final volume of 200 μ l. Supernatants were harvested after 24 hours and were stored at -20 °C.

Levels of IL-12p70 were determined in the supernatants by ELISA, using monoclonal antibodies 20C2 (Pharmingen) and biotinylated mouse-anti-human IL-12 C8.6 (Pharmingen) as coating and detection antibodies, respectively. Streptavidin-horseradish peroxidase (Sanquin) was used for detection, and additional ELISA reagents were from the PeliKine-Tool Set (Sanquin).

2.5 Determination of naïve Thelper cell polarization by DC

CD4⁺CD45RA⁺CD45RO⁻ naïve Th cells were purified from PBMC using a human CD4⁺/CD45RO⁻ column kit (R&D Systems). To determine T cell polarization, naïve T cells (4×10^4 cells/200 μ l) were cocultured with mature DC (1×10^4) in the presence of superantigen *Staphylococcus aureus* enterotoxin B (SEB, Sigma) at a final concentration of 100pg/ml in 96-well flat-bottom culture plates (Costar). At day 6, recombinant human IL-2 (10 u/ml, Strathman Biotech) was added and the cultures were expanded in 24 well culture plates (Costar). On day 12 the quiescent Th cells were restimulated with phorbol 12-myristate 13-acetate (PMA, 0.2 μ g/ml, Sigma) and ionomycin (2 μ g/ml, Sigma) in the presence of brefeldinA (20 μ g/ml, Sigma) during 5 hours. To detect intracellular cytokines, cells were fixed in 3.7% formaldehyde (Sigma) and stained in 0.5% saponin (Sigma) buffer using anti-human-IL-4-PE (BD Biosciences) and anti-human-IFN- γ -FITC (BD Biosciences), and were analyzed using a FACScan.

2.6 Statistical analysis

Data were analyzed for statistical significance using a paired t-test. Data were considered significant when the p value was less than 0.05.

3 Results

3.1 Isolation of monocytes from PBMC

PBMC were isolated from buffy coats by centrifugation on Ficoll, and were separated into lymphocytes and monocytes on a percoll gradient as described in Material and Methods. To monitor the purity of the monocyte fraction, FACS analysis was performed on isolated monocytes (Figure 2D-F) and unfractionated PBMC (Figure 2A-C). Live leukocytes were gated based on forward-sideward scatter plots (Figure 2A,D) and CD45 staining (Figure 2B,D) (CD45 is expressed on all human leukocytes). Within these gates, monocytes and T cells were identified by staining CD14, CD4 and CD8 (Figure 2C,F). CD14 is specifically expressed on monocytes, CD8 is expressed on cytotoxic T cells, and CD4 is highly expressed on T helper cells and moderately expressed on monocytes. Thus, monocytes appear as CD14⁺CD4^{low} in the FACS plots (lower right quadrant in Figure 2C,F), T cells are CD14⁻CD4⁺ or CD14⁻CD8⁺ upper left quadrant, and B cells are CD14⁻CD4⁻CD8⁻ (lower left quadrant). In the experiment shown in Figure 2, monocytes constituted 5% of the PBMC. After centrifugation on the percoll gradient as described in Material and Methods, the light density fraction contained 85% monocytes. Monocytes were further purified by plastic adherence: after 1 hour incubation at 37 °C/6% CO₂ the monocytes adhere to plastic, while the lymphocytes do not. To increase purity of the monocytes, non-adherent cells were washed away. After washing, purity of the monocytes is usually increased above 95% (J.H.N. Schuitemaker, personal communication). When monocytes develop into dendritic cells, they dissociate from the plastic and can easily be used for further studies.

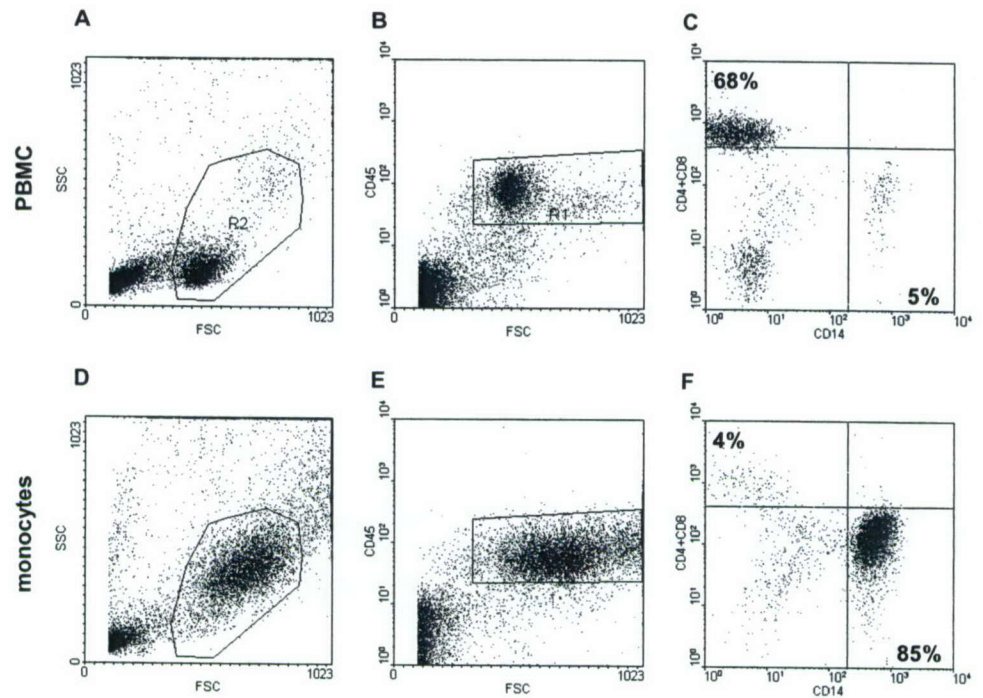


Figure 2 Isolation of monocytes from PBMC was monitored by FACS analysis of surface marker expression. A representative of 4 independent experiments is shown. Live leukocytes were gated as indicated in Figures A and B for PBMC and in Figures D and E for monocytes. In Figures C and F, only gated cells are shown.

3.2 Generation of immature DC

Adherent monocytes were cultured during 6 days in the presence of GM-CSF and IL-4 to generate immature DC. Generation of immature DC was monitored by FACS analysis of CD14 and CD1a expression; CD14 is expressed on monocytes but not on DC, while CD1a is expressed on DC but absent on monocytes. A representative FACS analysis is shown in Figure 3.

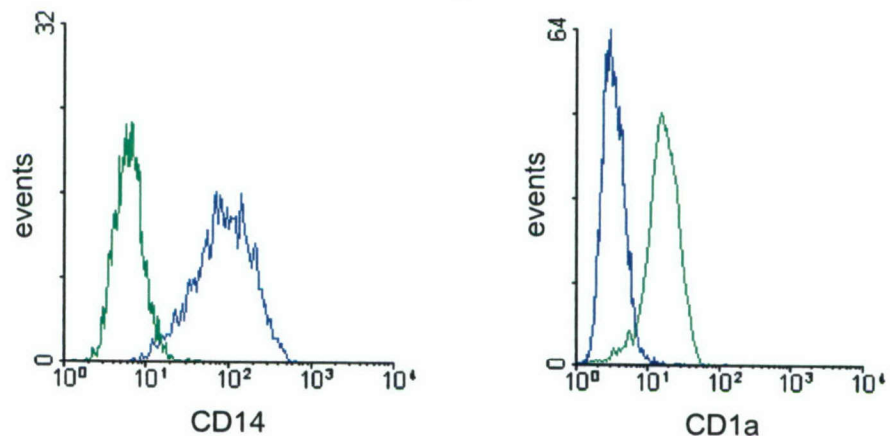


Figure 3 FACS analysis of monocytes (shown in blue) and immature dendritic cells (shown in green). A representative of 5 independent experiments is shown.

3.3 Maturation of DC

Maturation of DC was induced with LPS from *E.coli*. LPS was combined with IFN- γ to mature DC towards a phenotype that induces Th1 development, while LPS was combined with PGE2 to induce maturation of DC into a phenotype that promotes development of a Th2 response. Immature DC were stimulated with maturation factors during two days. After this incubation, maturation factors were thoroughly washed away, and maturation was analyzed by measuring expression of CD83, CD86 and HLA-DR by FACS analysis. Figure 4 shows the analysis of surface markers on immature and mature DC in different experiments. Although the expression levels of the surface markers are highly variable, expression of CD83, CD86 and HLA-DR is upregulated upon maturation in all experiments, which is in agreement with published findings by others [De Jong *et al.*, 2002].

Expression of CD83 was significantly upregulated upon addition of LPS ($p=0.010$), while there is no significant additional effect of addition of IFN- γ ($p=0.236$) or PGE2 ($p=0.109$). Expression of CD86 was also significantly upregulated upon addition of LPS ($p=0.0034$). However, for CD86, a significant additional upregulation was induced by IFN- γ ($p=0.035$). This additional upregulation of CD86 by IFN- γ has not been described before. PGE2 had no additional effect on CD86 expression ($p=0.8985$).

Expression of HLA-DR was significantly upregulated upon addition of LPS ($p=0.036$), while there is no significant additional effect of addition of IFN- γ ($p=0.333$) or PGE2 ($p=0.072$).

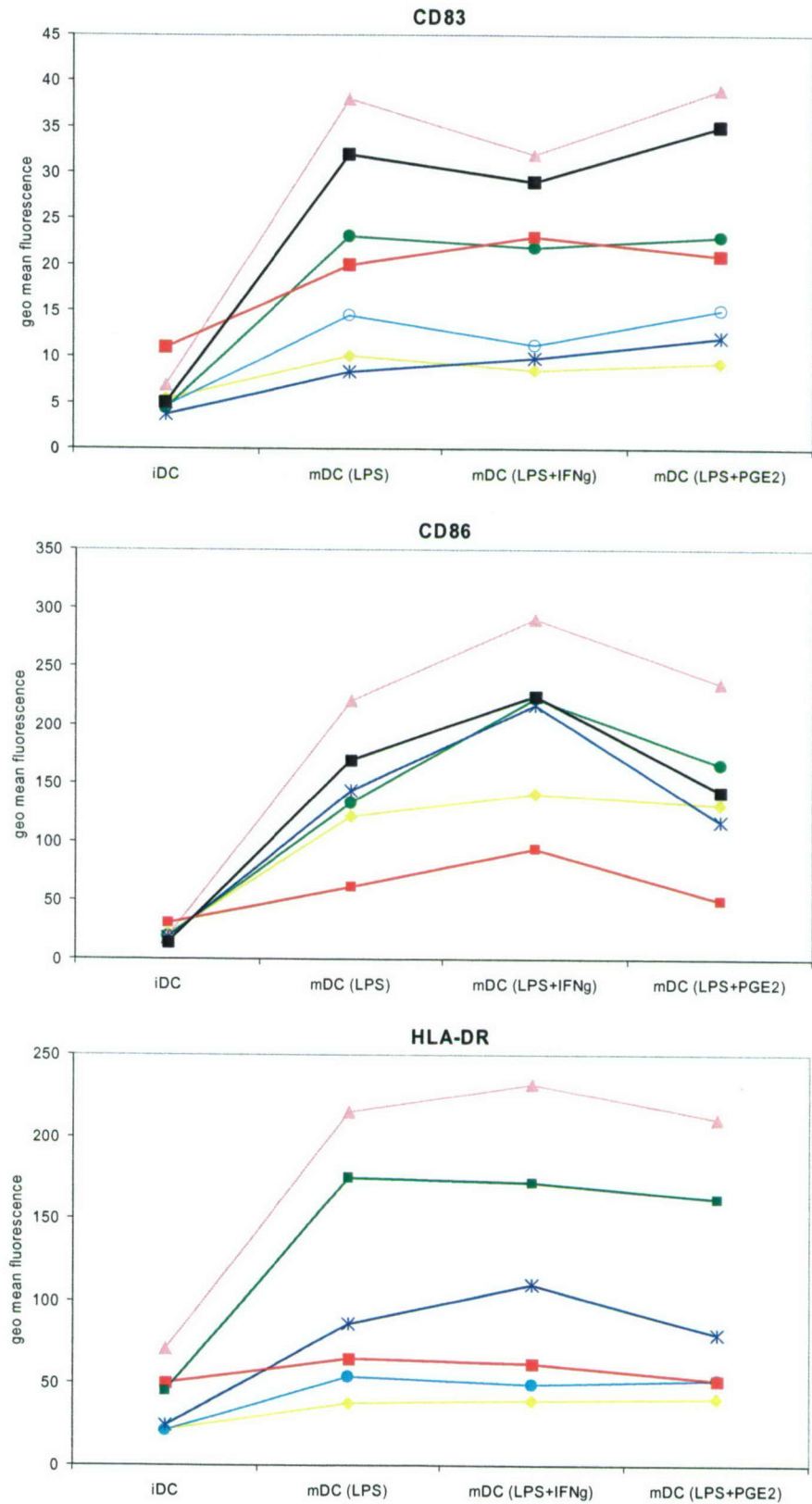


Figure 4 FACS analysis of surface marker expression of immature and mature DC. Results of independent experiments are indicated in different colours.

3.4 IL-12p70 production by DC

To mimic contact with T cells, mDC were co-cultured with a CD40L-transfected cell-line (J558 cells) during 24 hours. Levels of IL-12p70 were measured in the culture supernatants by ELISA. As can be seen in Figure 5, immature DC produce high levels of IL-12p70, which are downregulated upon maturation ($p=0.004$). In agreement with published findings, the presence of IFN- γ during maturation leads to enhanced production of IL-12 during CD40 ligation ($p=0.020$), while the presence of PGE2 during maturation decreases IL-12 production during CD40 ligation ($p=0.004$).

The degree of up- or downregulation of IL-12p70 strongly depends on the reagents used; culture medium, FCS or LPS from different suppliers give different results, although the same trends are observed (data not shown).

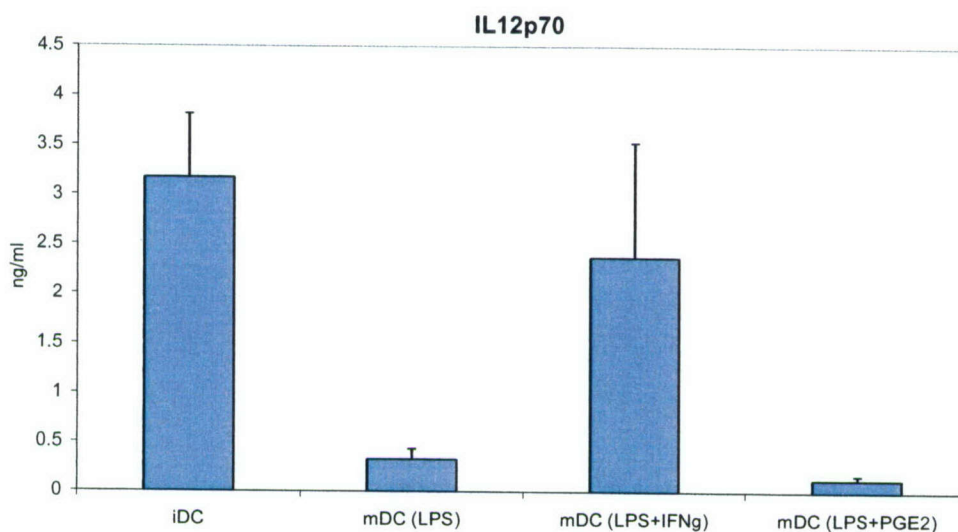


Figure 5 Production of IL-12p70 by DC upon CD40 ligation. Results of 5 independent experiments are presented as mean + standard deviation.

3.5 Polarization of T helper cell development by DC

To analyze the effects of the matured DC on development of acquired immunity, $1 \cdot 10^4$ DC were grown in the presence of $4 \cdot 10^4$ naïve T helper cells. During this co-culture, maturation factors (LPS, IFN- γ , PGE2) were no longer present. T cell proliferation was initiated by the mature DC. Six days after initiation of the co-culture, T cells were expanded by addition of IL-2, which is an essential growth factor for T cells. Twelve days after initiation of the co-culture, T cells were typically expanded to $2 \cdot 10^6$ - $5 \cdot 10^6$ resting T cells (resting cells are rounded off, while activated T cells are pear-shaped). To initiate cytokine production, resting cells were restimulated with PMA and ionomycin in the presence of brefeldinA, which blocks excretion of cytokines into the culture medium. To analyze T cell polarization, intracellular IFN- γ was stained as a typical Th1 cytokine, while intracellular IL-4 was stained as a Th2 cytokine. Staining of the cells was analyzed on a FACScan, as shown in Figure 6. Although the balance between Th1 and Th2

cells in the mixed response (initiated by LPS-treated DC) is variable between different donors, the amount of IFN- γ producing cells is always higher than the amount of IL-4 producing cells (this is in agreement with what is found by others, see for example De Jong *et al.*, 2002). As expected, the presence of IFN- γ during DC maturation resulted in DC that polarized T cell development towards Th1 (high percentage of IFN- γ producing cells, low percentage of IL-4 producing cells). On the other hand, the presence of PGE2 or Soluble Egg Antigen (SEA) of the parasitic worm *Schistosoma mansoni* during DC maturation gave rise to DC that polarized development of T cells towards a Th2 phenotype (elevated percentage of IL-4 producing T cells, low percentage of IFN- γ producing cells), which is also in agreement with findings by others [De Jong *et al.*, 2002].

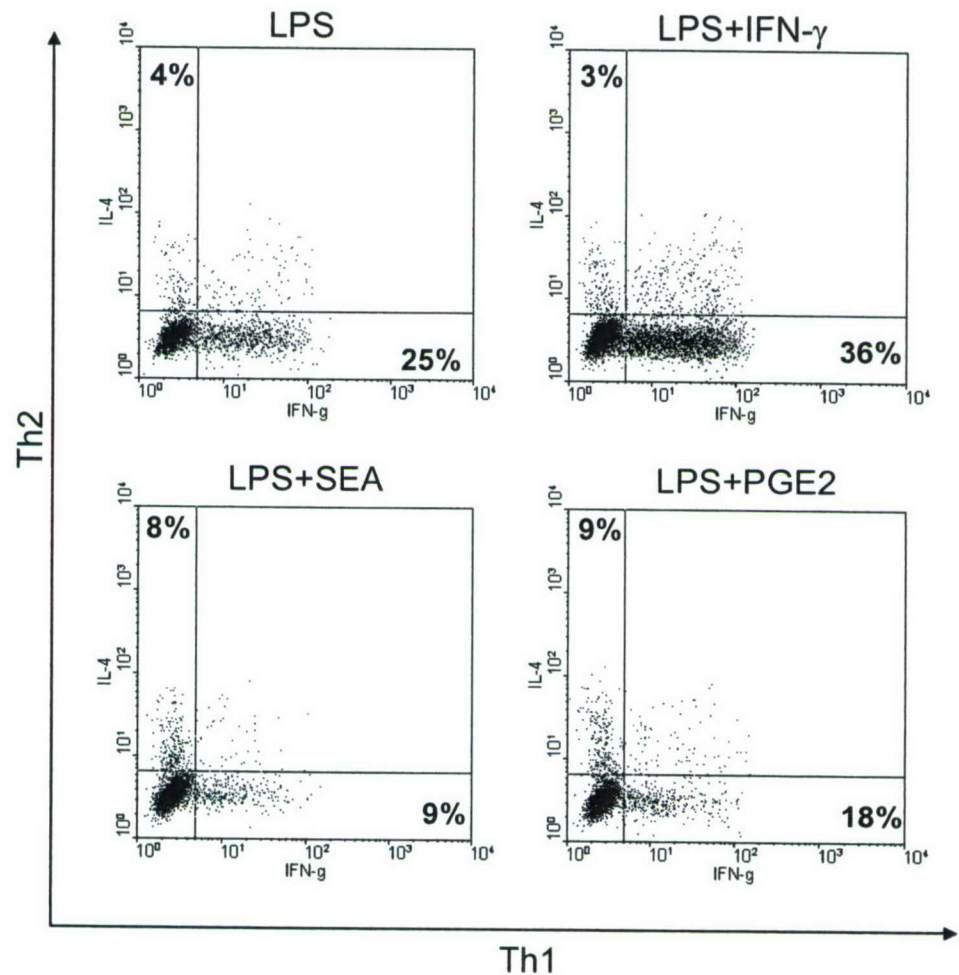


Figure 6 FACS analysis of T cell polarization induced by differently matured DC. Factors that were present during maturation of the DC are indicated on top of the plots. A representative of 5 independent experiments is shown.

4 Discussion and conclusions

4.1 Protection against biological warfare agents: a generic approach

Bacterial infections, amongst them those resulting from exposure to biological warfare agents, can be treated with antibiotics. However, because of the widespread use of these compounds, resistance of microorganisms against those antibiotics is becoming a large problem in the clinical setting. As a result, it might be possible that a bacterial infection cannot be treated efficiently with the antibiotic of choice and that precious time will be lost before a second, more efficient antibiotic compound can be administered.

In order to overcome these problems, vaccination of military personnel might be an option. However, it is not possible to develop vaccines against all possible threat agents. And even if this were feasible, it would not be wise to administer these vaccines to military personnel with the development of a possible Gulf War Syndrome in mind. But still, therapy is needed when military personnel is exposed to a biological warfare agent. A more generic approach to mitigate the effects of a broad spectrum of bacteria seems more effective.

In patients with cancer, the immune system is aspecifically stimulated with immunomodulators in addition to the treatment. It might be possible to use these compounds for a-specific stimulation of the immune system after an exposure to biological warfare agents.

4.2 The central role of dendritic cells in immune responses

Immunological defence against pathogens relies on both innate and adaptive immune responses. Invading pathogens are recognized by the innate immune system immediately upon infection. Recognition of pathogens by cells of the innate immune system is based on recognition of molecular patterns within a pathogen. This recognition is mediated by a battery of surface molecules, which include the Toll-like receptor (TLR) family [Akira et al, 2001]. For instance, TLR-2 binds to peptidoglycan of gram-positive bacteria like Streptococci and Staphylococci whereas TLR-4 binds to lipopolysaccharide (endotoxin) in the outer membrane of gram-negative bacteria like *Salmonella* and *E. coli*. TLR9 can bind to unmethylated CpG DNA motifs (highly represented in bacterial DNA, whereas host CpG islands are methylated), TLR5 to flagellin of motile bacteria like *Listeria* and TLR3 to double stranded RNA. Activation of the innate immune system subsequently leads to development of adaptive immunity through the activity of T helper cells. Adaptive immunity is specifically directed to the invading pathogen and forms immunological memory. In these two arms of the immune system, the dendritic cell (DC) has a special role: it forms the bridge between innate and adaptive immunity (illustrated in Figure 1). The DC itself belongs to the innate immune system, but upon activation initiates the formation of adaptive immunity. DC are present in an immature state in peripheral tissues as sentinels to detect pathogens immediately upon invasion. Exposure of DC to invading pathogens triggers a series of events involving antigen uptake and processing (maturation of DC) as well as migration to specialized lymphoid tissues for antigen presentation to T cells via so-called MHC molecules. In addition, activated DC generate signals that alert the immune system to potentially dangerous foreign material and modulate subsequent lymphocyte

activation and differentiation. Some of these signals are mediated by direct contact through costimulatory molecules on the DC surface such as CD40, CD80 and CD86, which are increased upon DC maturation, and others are mediated by cytokines and chemokines that are secreted by the DC. The combination of costimulatory molecules and cytokines that activates the T cells during contact with DC determines what type of adaptive immune response develops. Immunity against intracellular pathogens (viruses, bacteria) requires activity of Th1 cells, which activate macrophages and cytotoxic T cells, while defence against extracellular pathogens (parasitic worms, fungi) is mediated by Th2 cells, which activate eosinophils and stimulate antibody production by B cells.

4.3 Dendritic cells as an *in vitro* model for development of immune responses

The central role of the DC in the immune response makes this cell type highly suitable to study immune responses *in vitro*. Immature dendritic cells are found in blood, non-lymphoid and lymphoid tissues, whereas mature DC are primarily found in lymphoid tissues. In blood, two major types of DC have been described, myeloid and plasmacytoid DC. In addition, the corresponding precursors can be found, monocytes and pre-plasmacytoid DC, respectively. However, the amount of dendritic cells in blood is rather low, thus hampering the use of isolated dendritic cells as a model system to test immunomodulators. Therefore, many studies on DC make use of DC that are cultured from monocytes. Monocytes comprise about 5 to 20% of total white blood cells, and can therefore be isolated in reasonable quantities. When monocytes are cultured in the presence of Granulocyte/Macrophage – Colony Stimulating Factor (GM-CSF) and IL-4 for a week, the cells develop into immature DC. These immature DC can subsequently be exposed to pathogens or purified microbial molecules such as LPS, which will induce maturation of the DC.

The use of GM-CSF/IL-4-expanded monocytes as DC is widely accepted throughout the scientific community, and is based on three well-established and accepted criteria: first, their typical morphology (formation of dendrites), second, their surface phenotype, with expression of CD1, MHC class I and class II, CD80, CD40, ICAM-1, LFA-3 and CD11c, and third their high stimulatory response for naïve T cells (Sallusto and Lanzavecchia, 1994). Therefore, comparison between DC isolated from blood and DC generated *in vitro* from monocytes was not part of the study described herein.

This report describes the validation of a DC culture system at the TNO Defense, Security and Safety laboratory in Rijswijk, using the model compound LPS, which has well-described *in vitro* and *in vivo* effects on dendritic cells. Furthermore, for polarization, LPS was combined IFN- γ to induce maturation towards a DC phenotype that promotes development of Th1 cells, while LPS was combined with PGE2 or SEA of the helminth *Schistosoma mansoni* to induce maturation into DC that promote development of Th2 cells. The use of these model compounds was described earlier by De Jong *et al.* [2002], and is illustrated in Figure 7.

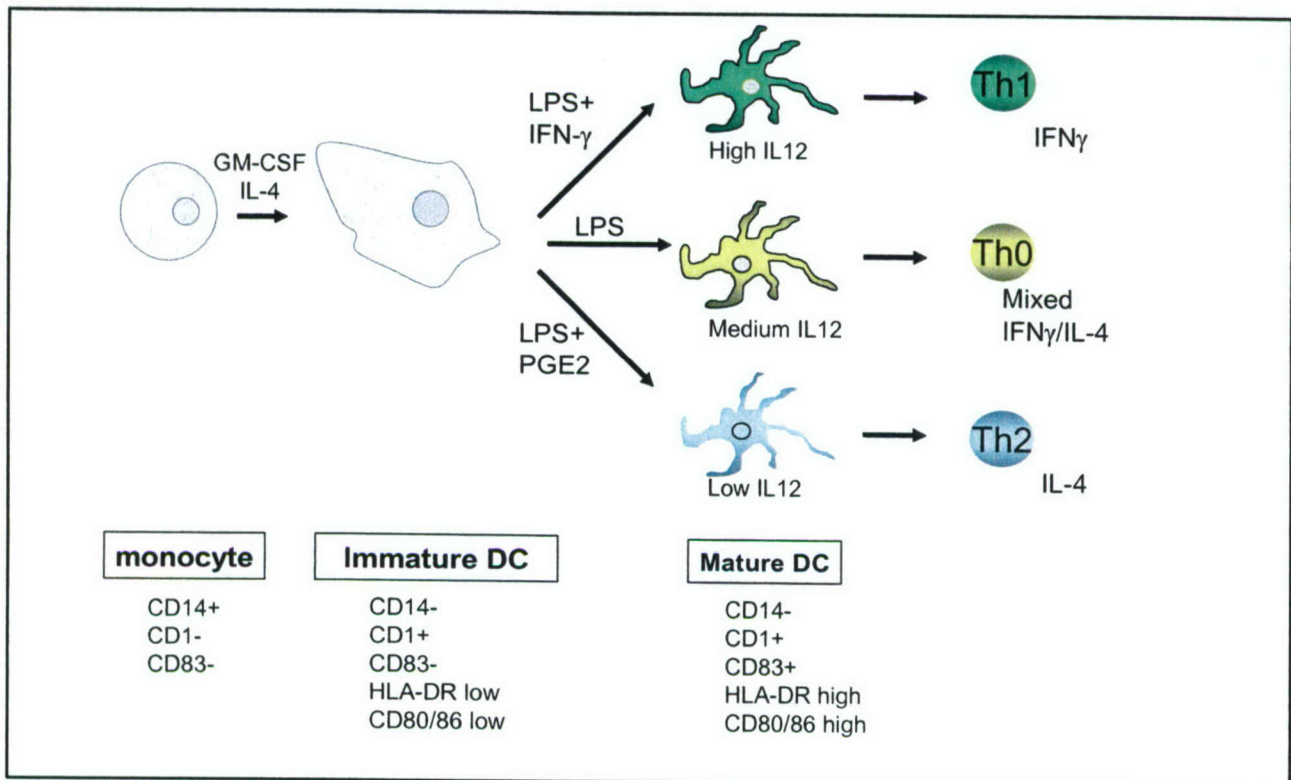


Figure 7 *In vitro* model of immune polarization by dendritic cells (DC). Immature DC are generated from monocytes, and are matured by lipopolysaccharide (LPS). Monocytes, immature DC and mature DC are characterized by the combination of surface markers indicated in the figure. Maturation of DC can be polarized towards Th1 or Th2 promoting DC by adding IFN- γ or PGE2, respectively. Polarized DC are characterized by the level of IL-12p70 they produce upon contact with T cells. Polarized T cells are characterized by the production of IFN- γ (Th1) or IL-4 (Th2).

4.4 Validation of the system

4.4.1 Isolation of monocytes

In the studies described in this report, monocytes were successfully isolated from buffy coats. By analyzing a combination of cell surface markers that could discriminate monocytes from lymphocytes through FACS analysis (Figure 2), we found that monocytes comprised between 5 and 15% of PBMC. This is in agreement with published findings. After enrichment by a Percoll gradient, purity of monocytes ranged from 70 to 90%. Monocytes were further purified through plastic adherence: at 37 °C monocytes readily adhere to the plastic surface of the culture wells, while lymphocytes float in the culture medium and can easily be removed.

4.4.2 Generation of immature DC

Immature DC were generated by culturing monocytes in the presence of GM-CSF and IL-4. Again, this development was checked by FACS analysis. Monocytes characteristically express CD14 but no CD1, while for DC the reverse is true. Indeed, as can be seen in Figure 3, culturing with GM-CSF and IL-4 abrogated CD14 expression and induced expression of CD1a. Thus, DC were successfully generated from monocytes. Maturation of DC is characterized by the induction of CD83 expression. Since CD83 expression was low or absent in these cells (Figure 4), DC were indeed in an immature state (CD14⁺CD1a⁺CD83⁻) after the initial culturing period with GM-CSF and IL-4.

4.4.3 *Maturation of DC*

Maturation of DC is induced when pattern recognition receptors on DC recognize microbial molecular patterns. As a model compound, we used lipopolysaccharide (LPS) from *Escherichia coli*. This commercial LPS preparation not only contains LPS but also some contaminating bacterial lipoproteins [Hirschfeld *et al.*, 2000]. Therefore, the preparation induces maturation of DC through activation of Toll-like receptor-4 by LPS and by activation of Toll-like receptor-2 by bacterial lipoproteins. Maturation of DC with LPS generates a DC phenotype that stimulates development of a mixed Th1/Th2 response. To include controls for polarization of DC maturation, LPS was combined with IFN- γ to mature DC towards a phenotype that induces Th1 development, while LPS was combined with PGE2 to induce maturation of DC into a phenotype that promotes development of a Th2 response. Maturation of DC was monitored by FACS analysis of CD83, CD86 and HLA-DR. CD83 is a maturation marker of which the function is still not known. CD86 is a co-stimulatory molecule that is essential for T cell activation by DC, and HLA-DR is an MHC molecule that presents microbial peptides to the T cell receptor on Thelper cells (see also paragraph 4.2). As expected, CD83, CD86 and HLA-DR were all significantly upregulated upon maturation. In addition, IFN- γ elevated CD86 expression even more. The latter is not in agreement with findings by others [De Jong *et al.*, 2002], which may result from the higher concentration of IFN- γ used in our study (5000u/ml in our study, 1000u/ml in the study of De Jong *et al.*). In both studies, DC in matured in the presence of IFN- γ were potent inducers of Th1 responses.

4.4.4 *Cytokine production by DC*

Cytokine production by DC may be determined at two different time-points: during the priming of DC by pathogens or during contact between DC and T cells. Although the first timepoint is important to attract other immune cells and to induce inflammation locally, the latter timepoint is far more relevant when it comes to developing effective immunity. The level of IL-12p70 (the bio-active form of IL-12) that is produced by DC during activation of naïve T cells is a major factor driving the polarization of the immune response: High levels of IL-12 result in Th1 priming, whereas Th2 responses are generated in the absence of IL-12. Therefore, we decided to measure cytokines that are produced by DC during contact with T cells. During this interaction, ligation of CD40 on the DC with CD40ligand (CD40L) on the T cell induces cytokine production in the DC. To mimic contact with T cells, DC were cultured in the presence of a cell-line that was transfected with CD40L. As can be seen in Figure 5, immature DC produce higher levels of IL-12 than mature DC. In agreement with published findings by De Jong *et al.* [2002], the presence of IFN- γ strongly upregulates IL-12 production by mature DC, while the presence of PGE2 leads to downmodulation of IL-12 production. This is in agreement with expectations, since IFN- γ is a Th1 priming stimulus while PGE2 primes for Th2 polarization.

4.4.5 *Polarization of T cell development*

To mimic the *in vivo* situation where DC come into contact with pathogens in the periphery and then meet the T cells in the lymph nodes where pathogens are absent, maturation factors were washed away before mature DC were incubated with naïve T cells. T cell proliferation was induced by the presence of DC. After an initial culturing period of six days, T cells were polyclonally expanded by adding the growth factor IL-2 to obtain sufficient numbers of T cells for further analysis.

Although immature DC produced substantial levels of IL-12, these DC did hardly induce T cell proliferation, probably as a consequence of low expression of costimulatory molecules on those DC (see Figure 4). For analysis of T cell polarization, production of IFN- γ (a Th1 marker, see Figure 7) and IL-4 (a Th2 marker) were analyzed by FACS. The balance between Th1 and Th2 cells in the mixed response (initiated by LPS-treated DC) is variable between different donors, and the number of Th1 cells is always higher than that of Th2 cells. Therefore, polarization cannot be determined by comparing absolute numbers of Th1 and Th2 cells, but rather by comparing polarized responses to the mixed situation in the 'LPS-treated DC'-group. In all experiments, the presence of IFN- γ during DC maturation lead to induction of a Th1 response, while the presence of PGE2 lead to a Th2 response. However, the effects of PGE2 on immune polarization were not always very clear, especially downmodulation of the percentage of IFN- γ producing T cells was not always detected. Therefore, a more 'natural' Th2 ligand, soluble egg antigen (SEA) of the helminth *Schistosoma mansoni*, which *in vivo* also induces Th2 responses, was tested as an alternative Th2 inducer. Fortunately, results with SEA were much more consistent. Therefore SEA will be used as a Th2 control in further studies.

Taken together, the *in vitro* DC culture system was successfully set up at TNO-Rijswijk, and was validated using the model compounds LPS, IFN- γ , PGE2 and SEA. The effects of these model compounds were in agreement with previous findings [Van der Kleij *et al.*, 2002] and with published studies by others [De Jong *et al.*, 2002] with respect to expression of surface markers, cytokine production and T cell polarizing capacity.

4.5 Future prospects

Now that the *in vitro* DC culture system is properly validated, this system will be used to monitor the effects of several bacterial species of military relevance on the immune system. Special attention will be given to possible immune evasion mechanisms that may be employed by these pathogens. Based on published findings, possible mechanisms include inhibition of DC maturation, downmodulation of costimulatory molecules, downmodulation of inflammatory cytokines (IL-12, TNF- α), induction of anti-inflammatory cytokines such as IL-10, induction of apoptosis in DC, and ineffective T cell priming [Agrawal *et al.*, 2003; Sing *et al.*, 2002]. When immune evasion mechanisms are identified that are used by several bacteria of military relevance, immunomodulators will be selected to counteract these mechanisms, and their effectiveness will be evaluated in the *in vitro* DC culture system.

4.6 Points of consideration

As can be seen in Figures 4 and 5, levels of surface markers or cytokines are highly variable between different donors. Therefore, to determine whether surface markers or cytokines are up- or downregulated by molecules or pathogens that are present during maturation, expression levels can only be compared within one experiment. To determine whether there is a true up- or downregulation, the experiment needs to be repeated at least three times to determine whether the observed effect is consistently present.

Figures 4 and 5 also show that maturation strongly influences expression of surface markers and production of cytokines. When pathogens instead of purified molecules are tested, each pathogen will induce maturation to a different level. To compare expression levels of surface markers or cytokines, all pathogens should be tested in various concentrations. In this way, one gets an impression of how efficient a pathogen induces maturation, or in other words, how many bacteria are required to induce full maturation (i.e. an expression level of CD83 equal to the level induced by LPS). In addition, active downmodulation of T cell stimulatory molecules and cytokine production can be assigned to a pathogen when CD83 expression is comparable to the LPS-matured DC, but other surface markers or cytokines are lower. Thus, effects of pathogens should be interpreted carefully, and only in the context of control compounds within the same experiment.

Many broad-spectrum therapeutics are ligands for TLRs. Human myeloid DC's express all TLRs with the exception of TLR9, which is selectively expressed by plasmacytoid DCs. Accordingly, plasmacytoid DCs respond to CpG DNA but not to LPS whereas myeloid DCs respond to LPS but not to CpG. Thus, depending on the characteristics of the type of immunomodulators, the model system must be chosen. Mostly, monocyte-derived DC can be used. However, in the case of ligands for the TLR9 like unmethylated CpG motifs, another system must be used, such as total PBMC stimulation.

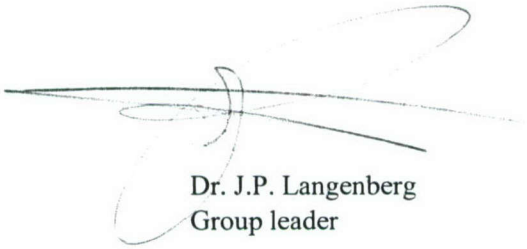
5 References

- Agrawal, A.; Lingappa, J.; Leppla, S.H.; Agrawal, S.; Jabbar, A.; Quinn, C. and Pulendran, B. (2003),
Impairment of dendritic cells and adaptive immunity by anthrax lethal toxin,
Nature. 2003 424(6946):329-34.
- Akira, S; Takeda, K. and Kaisho, T. (2001),
Toll-like receptors: critical proteins linking innate and acquired immunity,
Nature Immunology 2(8):675-80.
- Jong, E.C. de; Vieira, P.L.; Kalinski, P.; Schuitemaker, J.H.; Tanaka, Y.; Wierenga, E.A.; Yazdanbakhsh, M. and Kapsenberg, M.L. (2002),
Microbial compounds selectively induce Th1 cell-promoting or Th2 cell-promoting dendritic cells in vitro with diverse th cell-polarizing signals. *Journal of Immunology* 168(4):1704-1709.
- Hirschfeld, M.; Ma, Y.; Weis, J.H.; Vogel, S.N. and Weis, J.J. (2000),
Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2,
Journal of Immunology 165(2):618-22.
- Masihi, K.N. (2000),
Immunomodulatory agents for prophylaxis and therapy of infections,
International Journal of Antimicrobial Agents 14: 181-191.
- Masihi, K.N. (2001),
Fighting infection using immunomodulatory agents. *Expert Opinion on Biological Therapy* 1 (4): 641-653.
- Sallusto, F. and Lanzavecchia, A. (1994),
Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α ,
Journal of Experimental Medicine 179:1109-1118.
- Sing, A.; Rost, D.; Tvardovskaia, N.; Roggenkamp, A.; Wiedemann, A; Kirschning, C.J.; Aepfelbacher, M. and Heesemann, J. (2002),
Yersinia V-antigen exploits toll-like receptor 2 and CD14 for interleukin 10-mediated immunosuppression,
Journal of Experimental Medicine 196(8):1009-12.
- Kleij, D. van der; Latz, E.; Brouwers, J.F.H.M.; Kruize, Y.C.M.; Schmitz, M.; Kurt-Jones, E.A.; Espevik, T.; Jong, E.C. de; Kapsenberg, M.L.; Golenbock, D.T.; Tielens, A.G.M. and Yazdanbakhsh, M. (2002),
A novel host-parasite lipid cross-talk. Schistosomal lyso-phosphatidylserine activates toll-like receptor 2 and affects immune polarization,
Journal of Biological Chemistry 277(50):48122-9.

6 Signature

Rijswijk, May 2005

TNO Defence, Security and Safety



Dr. J.P. Langenberg
Group leader



Dr. D. van der Kleij
Author



Dr. M. Polhuijs
Author

ONGERUBRICEERD
REPORT DOCUMENTATION PAGE
 (MOD-NL)

1. DEFENCE REPORT NO (MOD-NL) TD2005-0029	2. RECIPIENT'S ACCESSION NO	3. PERFORMING ORGANIZATION REPORT NO DV2 2005-A029
4. PROJECT/TASK/WORK UNIT NO 014.12849	5. CONTRACT NO A01D4AL	6. REPORT DATE May 2005
7. NUMBER OF PAGES 24 (excl. RDP and distributionlist)	8. NUMBER OF REFERENCES 9	9. TYPE OF REPORT AND DATES COVERED Final
10. TITLE AND SUBTITLE Generic therapy after exposure to biological warfare agents: Validation of an <i>in vitro</i> dendritic cell culture system		
11. AUTHOR(S) Dr. D. van der Kleij, Dr. M. Polhuijs		
12. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) TNO Defense, Security and Safety, P.O. Box 45, 2280 AA Rijswijk, The Netherlands Lange Kleiweg 137, Rijswijk, The Netherlands		
13. SPONSORING AGENCY NAME(S) AND ADDRESS(ES) MGFB, P.O. box 20701, 2500 ES, The Hague, The Netherlands		
14. SUPPLEMENTARY NOTES The classification designation Ongerubricenseerd is equivalent to Unclassified		
15. ABSTRACT (MAXIMUM 200 WORDS (1044 BYTE)) The threat of the use of biological weapons, including bacteria, has increased. Bacterial resistance to antibiotics increasingly becomes a problem. Vaccination of military personnel against biothreat agents may be an option, however there is a broad range of biothreat agents, which may become even broader as a result of genetic engineering. Moreover, vaccination against multiple agents may cause undesired effects. A more generic approach to prevent the effects of a broad spectrum of bacteria via immunomodulation seems more effective. To be able to study the development of immune responses against bio-threat bacteria to select potential broad-spectrum therapeutics, an <i>in vitro</i> culture system of dendritic cells was chosen, since this cell type plays a central role in the development of immune responses. A dendritic cell culture system was validated using model compounds of which the effects on dendritic cells are known (LPS, IFN- γ , PGE2). The system was analyzed by measuring expression of surface markers on dendritic cells as well as cytokine production by dendritic cells and polarized T cells. All responses were in agreement with published literature in peer-reviewed journals.		
16. DESCRIPTORS		
Dendritic cell <i>In vitro</i> Cell culture	FACS Immune response Biological Weapons	IDENTIFIERS ELISA Cytokines
17a. SECURITY CLASSIFICATION (OF REPORT) Ongerubricenseerd	17b. SECURITY CLASSIFICATION (OF PAGE) Ongerubricenseerd	17c. SECURITY CLASSIFICATION (OF ABSTRACT) Ongerubricenseerd
18. DISTRIBUTION AVAILABILITY STATEMENT Unlimited Distribution	17d. SECURITY CLASSIFICATION (OF TITLES) Ongerubricenseerd	

ONGERUBRICEERD

Distributielijst

Onderstaande instanties/personen ontvangen het managementuittreksel en de distributielijst van het rapport.

- 4 ex. SC-DR&D
- 2 ex. Defensie R&D
Hoofd Cluster 2 KTZE J.Wind
- 1 ex. TNO Defensie en Veiligheid, Algemeen Directeur,
ir. P.A.O.G. Korting
- 3 ex. TNO Defensie en Veiligheid, Directie
Directeur Operaties, ir. C. Eberwijn
Directeur Kennis, prof. dr. P. Werkhoven
Directeur Markt, G.D. Klein Baltink
- 1 ex. TNO Defensie en Veiligheid, accountdirector CO
- 1 ex. TNO Defensie en Veiligheid, vestiging Den Haag,
Manager Business Unit Waarnemingssystemen (operaties),
dr. M.W. Leeuw
- 1 ex. TNO Defensie en Veiligheid, vestiging Den Haag,
Manager Business Unit Beleidsstudies Operationele Analyse & Informatie
Voorziening (operaties), drs. T. De Groot
- 1 ex. TNO Defensie en Veiligheid, vestiging Rijswijk,
Manager Business Unit Bescherming, Munitie en Wapens (operaties),
ir. P.J.M. Elands
- 1 ex. TNO Defensie en Veiligheid, vestiging Soesterberg,
Manager Business Unit Gedrag, Training & Prestatie (operaties),
drs. H.J. Vink
- 1 ex. TNO Defensie en Veiligheid, vestiging Soesterberg ,
Communicatiemanager TNO Defensie en Veiligheid,
P.M. van Bergem-Jansen
- 1 ex. Lid Instituuts Advies Raad D&V
BGen. prof. J.M.J. Bosch
- 1 ex. Lid Instituuts Advies Raad D&V
prof. dr. U.A. Th. Brinkman
- 1 ex. Lkol L. Jobse
DS/DOBBP
- 1 ex. It-kol mr. R.C. Nulkes
DS/IMS/Afd. Navo-EU/Stafofficier Navo, nucleaire en non-
proliferatiezaken
- 1 ex. D.M. van Weel
HDAB
- 1 ex. Drs. E.S.A. Brands
DJZ/IJB

- 1 ex. Lkol A. Solkesz
HDP/DPB
- 1 ex. Maj. R.F.M. Schröder
Las/PBDL/OB
- 1 ex Maj. R. Brunsting
DS/DOBBP/Operationele Behoeftestelling
- 1 ex H.G.B. Reulink
LBBKL/KPU-bedrijf

Onderstaande instanties/personen ontvangen een volledig exemplaar van het rapport.

- 1 Defensie R&D
T.a.v. ir. P.J. Keuning
- 2 Referent
KLTZE E.H. Nieuwenhuis
- 3 Programmabegeleider V013,
CDC/MGFB, A.S. de Koning
- 4 MGFB,
Dr. H.J. Jansen, Projectbegeleider
- 5 DMG
Lkol B. Lam
- 6 DMG
KLTZAR N.I. van Zaalen Boelema Robertus
- 7/8 DMIVD,
Kabinet
- 9 CJKC NBC,
Lkol C.A. Bourgondiën
- 10 Programmaleider V013, dr. ir. M. S. Nieuwenhuizen
- 11 TNO Defensie en Veiligheid, vestiging Rijswijk,
Manager Business Unit BC Bescherming (operaties), ir. R.J.A. Kersten
- 12 TNO Defensie en Veiligheid, vestiging Rijswijk,
Manager Business Unit BC Bescherming (kennis), dr. R.W. Busker
- 13 TNO Defensie en Veiligheid, vestiging Rijswijk,
Manager Business Unit BC Bescherming (markt), ir. G.C. de Valk
- 14/15 TNO Defensie en Veiligheid, vestiging Rijswijk,
Informatie- en Documentatiedienst
- 16/18 TNO Defensie en Veiligheid, vestiging Rijswijk,
Business Unit BC Bescherming, dr. J.P. Langenberg, dr. M. Polhuijs,
dr. D. van der Kleij

- 19 TNO Defensie en Veiligheid, vestiging Rijswijk,
Marketing en Communicatie, digitale versie via Archief
- 20/22 Bibliotheek KMA