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TNO report

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**Boosting immune responses against
bacterial pathogens: in vitro analysis
of immunomodulators**

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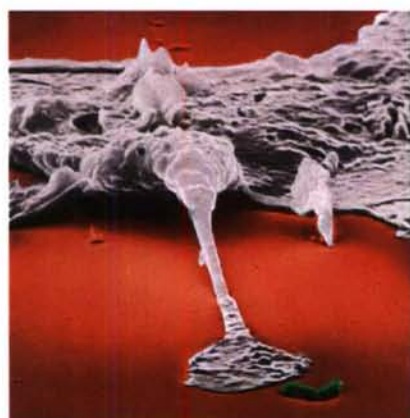
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In vitro analyse van de stimulerende werking van verschillende stoffen op het immuunsysteem

Het onderzoek beschrijft de in vitro analyse van de stimulerende werking van verschillende stoffen op het immuunsysteem. Door het immuunsysteem in een bepaalde richting te stimuleren kan de afweerreactie tegen bacteriën worden versterkt. Hierdoor zouden de gevolgen van een infectie met biologische strijdmiddelen mogelijk kunnen worden beperkt.



Probleemstelling

Diverse ontwikkelingen wereldwijd 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 behandelen met antibiotica. Door spontane of opzettelijk geïntroduceerde 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 relatief eenvoudige wijze een grote verscheidenheid aan biologische wapens te produceren waartegen bestaande vaccins of antibiotica niet meer werkzaam zijn. Bovendien bestaan tegen virusinfecties nagenoeg geen therapeutische opties. Het is onmogelijk om tegen al deze producten de

afzonderlijke vaccins te ontwikkelen.

Ook is het niet wenselijk om zoveel vaccins tegelijk toe te dienen gezien de onbekendheid met mogelijke interacties. Om deze redenen lijkt een meer generieke aanpak waarmee de effecten van een groot scala aan bacteriën kan worden voorkomen, een effectievere aanpak. Bij kankerpatiënten wordt reeds getracht het afweersysteem specifiek te stimuleren door middel van het toedienen van immuno-modulatoren, stoffen die het immuunsysteem activeren. Immunomodulatoren zouden mogelijk ook kunnen worden toegepast om het afweersysteem van militairen op niet-specifieke wijze te stimuleren om de afweer tegen een breed scala aan bacteriën en virussen te versterken.

In opdracht van het Ministerie van Defensie wordt de haalbaarheid van deze aanpak onderzocht door TNO. Dit onderzoek is uitgevoerd door TNO Defensie en Veiligheid, locatie Rijswijk. Het onderzoek is gefinancierd in het kader van het programma V502 en beantwoordt aan deliverable nummer 7.2.1.

Beschrijving van de werkzaamheden

De effecten van combinaties van verschillende immuunmodulerende stoffen werden bepaald in een relevant in vitro-model, te weten een kweekstelsel van dendritische cellen. Een kweekstelsel van dendritische cellen werd blootgesteld aan drie verschillende stoffen (MPL, MDP en

ssPolyU) die het immuunsysteem kunnen moduleren, al dan niet gecombineerd met pathogenen, waaronder *Yersinia pestis*, *Vibrio cholerae* en MRSA.

De immuunmodulerende werking werd geanalyseerd door de aanwezigheid van moleculen op het celoppervlak en de productie van signaalmoleculen te meten.

Resultaten en conclusies

De drie geteste modulatoren bleken de immuunactiverende werking van dendritische cellen te verhogen, en elkaars werking hierbij te versterken.

Bovendien bleken twee modulatoren en hun combinatie de immunrespons die vervolgens door de gemoduleerde dendritische cellen in gang werd gezet, te versterken in de zogenoemde Th1-richting, wat de respons is die van belang is bij de bestrijding van bacteriële infecties. Deze twee modulatoren en hun combinatie zullen vervolgens in een in vivo-infectiemodel in de muis worden getest.

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 immunomodulatoren uitkomst kunnen bieden. Door het niet-specifiek stimuleren van het afweersysteem door deze therapeutica

zouden de effecten van blootstelling aan een groot scala aan biologische agentia (bacteriën) mogelijk kunnen worden geminimaliseerd.

Daarnaast zouden immunomodulators kunnen worden toegepast om infecties te voorkomen (profy lactische toepassing). In het bovengenoemde onderzoek is de reactie van dendritische cellen op enkele immunomodulators in kaart gebracht. Op basis van de verkregen resultaten zijn twee modulators geselecteerd om *in vivo* in een muismodel te testen als therapeutica.

Ongewenste effecten van specifieke stimulatie van het immuunsysteem zouden condities kunnen zijn waarbij het immuunsysteem te actief is, zoals het geval is bij auto-immuunziekten. In recent onderzoek naar de veiligheid van immunomodulators die vallen in dezelfde categorie als de modulators die in het huidige onderzoek worden beschreven, werden echter geen bijwerkingen gevonden (voor een uitgebreide beschrijving zie rapport TNO-DV2 2005 A205).

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Summary

Bacterial infections, including 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 significant problem in the clinical setting. As a result, it may be possible that a bacterial infection cannot be treated efficiently with the antibiotic of choice and that precious time is lost before a second, more efficient antibiotic compound can be administered. In addition, viral infection nowadays cannot be efficiently treated.

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 desirable to administer all these vaccines at the same time to military personnel, since the possible interactions between different vaccines are unknown. 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.

In the studies described in this report, the effects of three immunomodulators (MDP, MPL and ssPolyU) and their combinations were tested in vitro in a culture system of human dendritic cells. Dendritic cells play a central role in the coordination of developing immune responses. Upon pathogen encounter in peripheral tissues, dendritic cells undergo a maturation process and migrate towards the lymph nodes, where they orchestrate the development of immunity via Thelper cells. The response of the dendritic cells to immunomodulators in vitro was analyzed by measuring the expression of surface molecules on dendritic cells as well as cytokine production by dendritic cells and polarized T cells. The effects of the modulators on dendritic cells were determined in the absence or presence of pathogens.

The response to pathogens may be enhanced either by enhancing dendritic cell maturation, or by increasing the Thelper type 1 cell driving capacity of dendritic cells (DC). When immunomodulators were tested in the absence of pathogens, DC maturation was synergistically enhanced when the modulator MDP was combined with a TLR4 or a TLR8 ligand, while the two TLR ligands did not act synergistically. Furthermore, the immunomodulators did not modify the T cell polarizing capacity of dendritic cells in the absence of pathogens. This might indicate that these modulators could be used profylactically, since they will only exert their immune-polarizing effects when an infection occurs.

When the immunomodulators were tested in the presence of pathogens, their effect on dendritic cell maturation was more pronounced when combined with pathogens that are weak inducers of dendritic cell maturation. Considering the modulation of T cell polarizing capacity, the modulators MDP and ssPolyU and their combination enhanced Th1 driving capacity of dendritic cells when combined with some but not all pathogens, while MPL and combinations with MPL did not modify T cell polarization in this direction.

Based on the results of this in vitro study, MDP combined with a TLR8 ligand will be tested in an in vivo mouse infection model.

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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 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 is lost before a second, more efficient antibiotic compound can be administered.

Resistent *Yersinia pestis* strains, most likely due to spontaneous mutation, were discovered in Madagascar. In addition, it is assumed that within the former so-called 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 desirable to administer these vaccines to military personnel, since the possible interactions between different vaccines are unknown. 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. It might be possible to use these compounds for aspecific stimulation of the immune system after an exposure to biological warfare agents.

1.2 Dendritic cells: natural modulators of the immune response

Dendritic cells (DC) are central players in the formation of immune response. 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 receptors which mediate recognition of microbial molecules. Upon activation through these receptors, 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.

Despite the fact that innate immune recognition has evolved as a defence strategy against pathogens, many pathogens in turn have evolved strategies to manipulate the immune system of their host to enhance survival of the pathogen. Several BW-bacteria are now known to employ such evasive strategies (for more details see TNO report DV2-2005-A204). To counteract these effects, immunomodulators may be used to make the immune system more effective.

To be able to monitor the effects of pathogens on the host system, an *in vitro* test system based on dendritic cells was developed, as described in TNO report TNO-DV2 2005-A029. The effects of possible BW-bacteria in this dendritic cell culture system was described in TNO report TNO-DV2 2005-A204. The current report describes the effects of the first immunomodulators tested in this dendritic cell culture system.

1.3 Modulation of dendritic cells: modulating the modulators

Given the central role of dendritic cells in the developing immune response, and their steering role in this process, dendritic cells are an attractive target for immunomodulatory therapies.

As stated above, dendritic cells vigorously respond to pathogens. Therefore, molecules derived from pathogens are obvious candidates as immunomodulators. The use of microbial products to enhance the immune response against pathogens has been applied for some time now in the context of vaccinations: in addition to inactivated pathogens or pathogen subunits, vaccines also contain so-called adjuvants. These adjuvants usually are composed of microbial products (derived from other pathogens) that are known to enhance immunity. Traditional adjuvants often gave rise to many side-effects upon administration. Current research on adjuvants for vaccines is aimed at enhancing the effectivity and specificity of adjuvants while reducing the side-effects.

An important development in the field of immunity against infections is the identification of so-called 'Toll-like receptors' (TLRs). In humans, 11 different receptors of this family have been identified so far. Each of these receptors is activated by defined molecules that are present in/on pathogens but absent in humans. These molecules are of essential importance for survival or pathogenicity of the pathogen, and have therefore been strongly conserved throughout evolution. Since the identification of TLRs and the identification of their essential role in the initiation of immune responses against pathogens, TLRs have been a topic of intense research.

At the moment, research on the possibilities to intervene in the activation cascades of TLRs is being carried out in laboratories throughout the world, with the aim to strengthen the immune response without inducing sepsis. For example, certain molecules that activate TLR9 have been shown to act as a potent adjuvant in vaccinations against hepatitis B, and to enhance the effects of chemotherapy against cancer. Although most research on the therapeutic possibilities of TLR ligands is concentrated on their applicability as vaccine adjuvants, some studies include data on

the effects of TLR ligands as stand-alone agents, both in prophylactic and in therapeutic settings.

In addition to TLRs, other pattern recognition receptors are also involved in the recognition of invading pathogens. A recently identified family of cytoplasmic pattern recognition receptors is the NOD-like receptor (NLR) family. One of the major roles of NLRs is to detect microbial molecules that are present in the cytoplasm of cells. One of the first mammalian NLRs reported to recognize microbial molecules is NOD2. NOD2 recognizes muramyl dipeptide (MDP) and acts as a general sensor for most bacteria. Although NOD2 has been identified only recently, the effect of its ligand MDP as an adjuvant when combined with vaccines has been documented for quite some time.

Recent publications have shown that various combinations of TLRs may synergize in the induction of a particular set of genes in dendritic cells. For example, TLRs that use the adaptor molecule TRIF (TLR3 and TLR4) have been shown *in vitro* to synergize with TLRs that are present intracellularly in endosomes (TLR7, TLR8 and TLR9) to increase the production of the Th1-polarizing cytokine IL-12p70 [Napolitani et al, 2005; Gautier et al, 2005]. TLR interplay seems to be important for at least certain infections *in vivo*, because mice defective in both TLR2 and TLR9 are much more susceptible to aerosol mycobacterial infection than mice that are defective in either TLR [Bafica et al, 2005].

NLRs have also been reported to act in synergy with TLRs *in vitro*. For example, in monocytes and DC, NOD1 and NOD2 agonists act cooperatively with LPS (activating TLR4) to stimulate the production of inflammatory cytokines such as TNF- α and IL-6; they can also synergize with subthreshold doses of LPS to induce DC maturation [Fritz et al, 2005]. NOD1 and NOD2 also act in synergy with TLR3, TLR4 and TLR9 (but not TLR2) in DC to induce IL-12p70 production and promote Th1 differentiation [Tada et al, 2005].

In the studies described in this report, combinations of NLR and TLR ligands have been tested in a dendritic cell culture system to evaluate their potency as immunomodulators that may enhance immunity against bacterial infections. Based on the observation described above that TRIF-coupled TLRs and endosomal TLRs act in synergy, MPL and ssPolyU were tested as TLR4 and TLR8 ligands, respectively. Lipopolysaccharide (LPS), the most well-known activator of TLR4, has long been known to enhance innate and adaptive immune responses; however, its extreme toxicity precludes its use in clinical settings. Therefore, less toxic derivatives have been developed, such as monophosphoryl-lipid A (MPL). MPL has been shown to activate TLR4, and is extensively used as a vaccine adjuvant. ssPolyU is a single-stranded poly-uridine oligonucleotide that is recognized by human TLR8 and murine TLR7.

In addition, the NOD2 ligand MDP was also included in the study. MDP is the minimal peptidoglycan motif common to all bacteria, an essential structure required for adjuvant activity of well-known adjuvants in vaccinology. Combinations of these ligands were analyzed for their capacity to (1) enhance DC maturation and to (2) enhance Th1 stimulatory capacity of DC.

2 Material and methods

2.1 Bacterial strains, growth conditions and inactivation

The bacterial strains used are summarized in Table 1. Bacteria were grown at 37 °C for 18 h in Brain Heart Infusion (BHI) medium. For *Yersinia* cultures the BHI medium was supplemented with 2.5 mM CaCl₂ to keep the KIM5 plasmid in the bacteria.

For UV-inactivation of bacteria the overnight cultures were centrifuged at 4500 rpm for 30 min. Pellets were resuspended in 5 ml physiological salt solution and irradiated with shortwave UV energy (254 nm) with a UV crosslinker (UVP). The amount of UV energy needed to inactivate the bacteria is listed in Table 1. To confirm the inactivation, 100 µl of the irradiated bacterial suspension was plated on a TSA plate and incubated for 72 hours under appropriate growth conditions. After inactivation, bacteria were quantified by FACS using a BD Cell Viability kit with coating beads (BD Biosciences).

Bacterial strain	NCTC/ ATCC	UV Energy (µJoule/cm ²)
<i>Bacillus anthracis</i>	NCTC 10340	3 000
<i>Escherichia coli O157:H7</i>	HLO/ Hospital	3 000
<i>MRSA</i>	HLO/ Hospital	10 000
<i>Vibrio cholerae</i>	Slotervaart Hospital	1 000
<i>Yersinia pestis KIM-5</i>	FOI	18 000

Table 1 Bacteria used in this study, source and energy dose required for inactivation.

2.2 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 (1750 g) 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*10⁵ 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, 500 u/ml recombinant GM-CSF (a kind gift from Joost Schuitemaker, AMC, Amsterdam) en 250 u/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.

2.3 Induction of maturation of iDC

At day 6 or day 7, maturation of iDC was induced by the addition of 100 ng/ml LPS from *E.coli* O55:B5 (Sigma). For polarization studies, recombinant IFN-γ (1000u/ml, Strathmann Biotech) was added in combination with LPS to induce Th1-promoting DC, while Soluble Egg Antigen (SEA, 25 µg/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. Bacteria were added to DC to a final concentration of 10^6 bacteria/ml, unless stated otherwise. For immune modulation, muramyl dipeptide (100 ng/ml), ssPolyU (1 μ g/ml) and monophosphoryl lipid A (2.5 μ g/ml) were from Invivogen.

2.4 Analysis of expression of cell surface markers

Expression of cell surface markers was analyzed using mouse anti-human monoclonal antibodies against CD14-PerCP (BD Biosciences), CD11a-PE (Immunotech), CD83-PE (Immunotech), HLA-DR-FITC (BD Biosciences), CD80-PE (BD Biosciences) and CD86-FITC (BD Biosciences), and was analyzed using a FACScan (BD Biosciences).

2.5 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). Levels of TNF- α and IL-10 were measured in supernatants using commercial kits (Sanquin) following the manufacturer's recommendations.

2.6 Determination of naïve T helper 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.

2.7 In vitro stimulation of murine splenocytes

Primary splenocytes were isolated from the spleen of non-infected mice (chrySTALLIN, male), and were seeded in 96-well flat-bottom culture plates (Costar). Cells were stimulated with MDP, murabutide, CLO75 and ODN1826 (all from Invivogen) in a final volume of 200 μ l. Supernatants were harvested after 24 h, and interleukin-6 concentrations were determined using a mouse IL-6 ELISA Ready-Set-Go kit (eBioscience).

2.8 Statistical analysis

Data were analyzed for statistical significance using an unpaired t-test. Correlations were calculated using Pearson correlations using two-tailed p-values. Data were considered significant when p values were less than 0.05.

3 Results

3.1 Effects of modulators on DC maturation

To monitor the effects of the modulators on the maturation of DC, immature DC were cultured in the presence of MPL, MDP, ssPolyU or combinations of these modulators. After two days, expression of surface molecules on DC was measured using FACS to assess the level of maturation of the cells. As can be seen in Figure 1, MPL induced maturation of DC: CD83, HLA-DR, CD80 and CD86 were all upregulated. ssPolyU and MDP had no effect on DC maturation when used as stand-alone agents. However, when modulators were combined, MDP enhanced maturation when combined with MPL or ssPolyU. Thus, MDP can act in a synergistic manner with both TLR ligands. The levels of the markers induced varied between experiments, however the same trends were observed in all experiments.

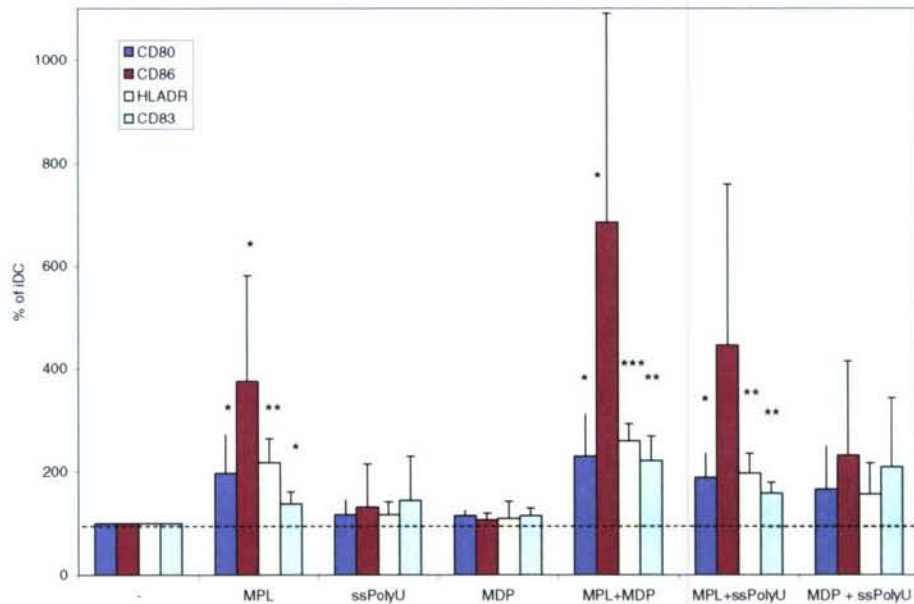


Figure 1 Surface molecules on DC upon stimulation with immunomodulators, measured by FACS analysis. The mean+SD of 3 independent experiments is shown, the expression on immature (non-modulated) DC was set at 100%. Statistically significant enhancement of expression as compared to non-modulated DC is indicated (*:p<0.05, **:p<0.01, ***:p,0.001).

To study whether the modulators could enhance maturation of DC that was induced by pathogens, DC were exposed to various inactivated bacteria (*Y.pestis*, *V.cholerae*, *B.anthraxis*, MRSA and *E.coli* O157:H7) in the presence or absence of the modulators mentioned above. To be able to study the effects of the modulators, a concentration of bacteria (10^6 bact/ml) was used that did not induce full maturation (for details see TNO report TNO-DV2 2005-A204). The effects of the bacteria on DC maturation is shown in Figure 2, and the effects of the modulators on top of these pathogen-induced effects are depicted in Figure 3. In agreement with previous findings (see report TNO-DV2 2005 A204), gram negative bacteria induced higher levels of maturation than gram positive bacteria. *E.coli* induced the highest level of maturation (Figure 2).

In Figure 3, the maturation levels shown in Figure 2 were set at 100% to see the extra maturation induced by the modulators. The effect of the modulators appeared to vary when different pathogens were used. In general, the effect of the modulator(s) was more prominent when combined with pathogens that were weak inducers of maturation. This inverse correlation is shown in Figure 4 for the modulator combination MDP+ssPolyU (correlations are considered to be statistically significant when $p < 0.05$). As illustrated in this Figure, when a pathogen induced high levels of surface molecules, these levels were only marginally upregulated further by the MDP+ssPolyU combination. In contrast, when a pathogen induced low levels of surface molecules by itself, the addition of MDP+ssPolyU increased the expression levels of the surface molecules significantly.

When considering the modulators, MDP as stand-alone agent did not have much effect on the level of maturation, as was also the case in the absence of pathogens. In contrast, ssPolyU and MPL in combination with most pathogens enhanced maturation both as stand-alone agents and in combination with other modulators (Figure 3).

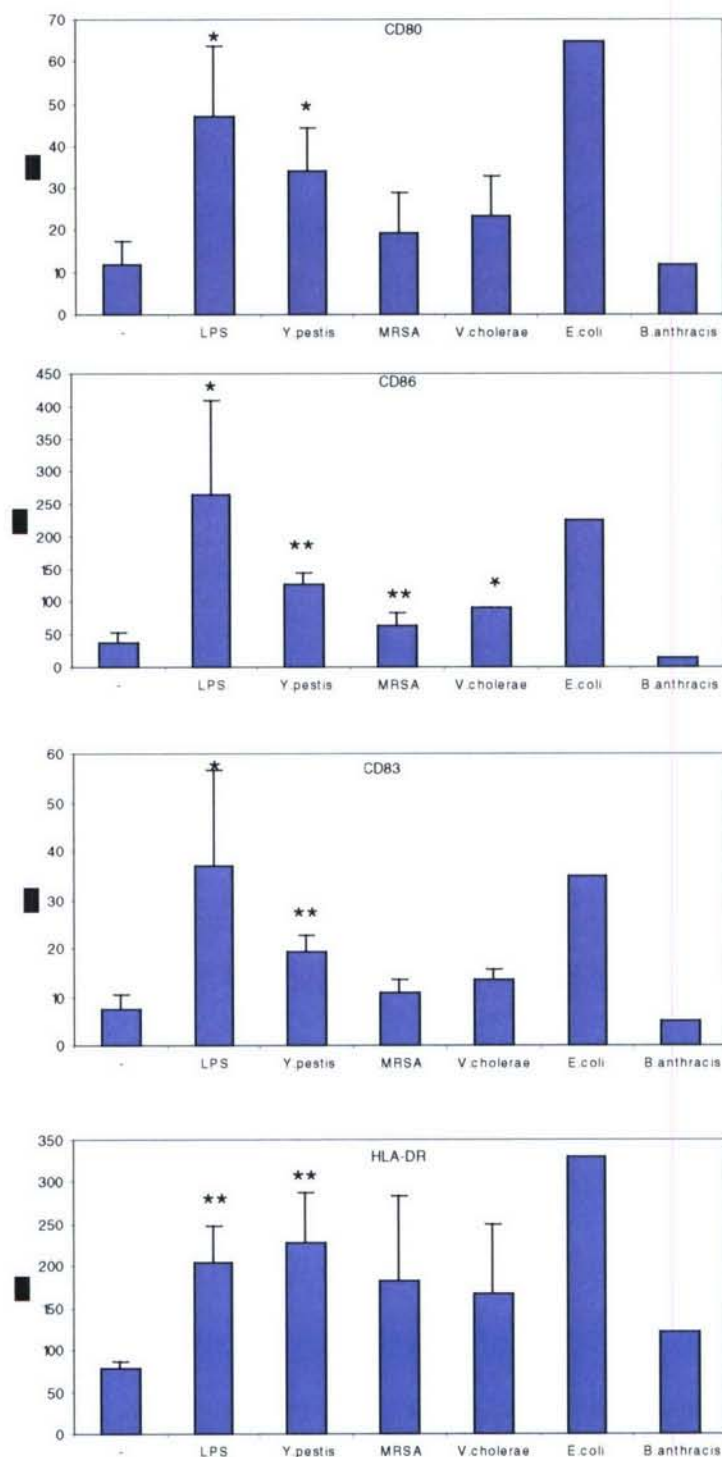


Figure 2 Surface molecules on DC upon stimulation with pathogens (indicated as mean fluorescence intensity (MFI), measured by FACS analysis. LPS was used as a positive control of DC maturation. The mean+SD is shown. *Y.pestis* and MRSA were tested in 3 independent experiments, *V.cholerae* in 2 independent experiments, and *E.coli* and *B.anthraxis* in a single experiment. Statistically significant enhancement of expression as compared to non-modulated DC is indicated (*:p<0.05, **:p<0.01).

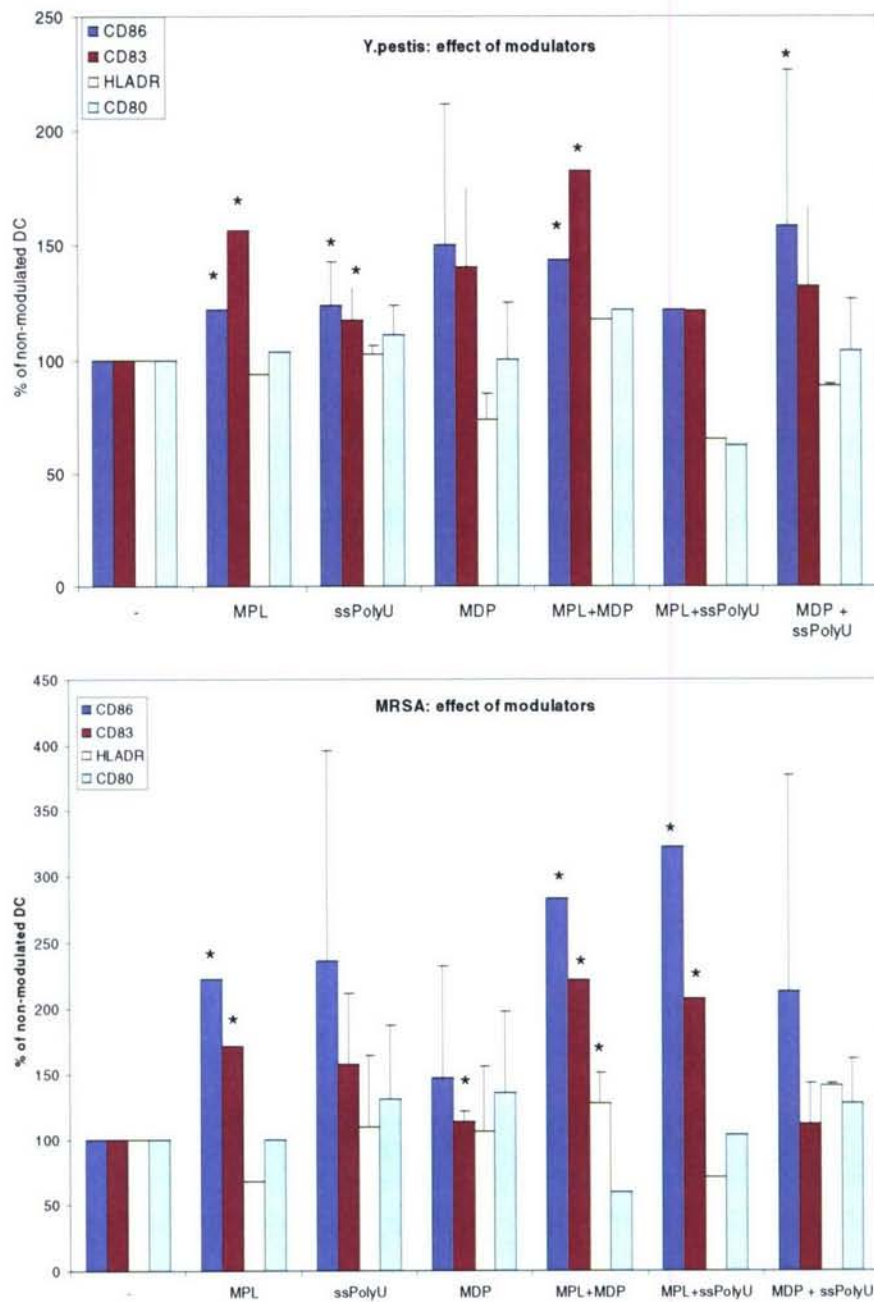


Figure 3A Surface molecules on DC upon stimulation with pathogens in combination with immunomodulators, measured by FACS analysis. The mean+SD of 3 independent experiments is shown, the expression on DC exposed to the respective pathogen in the absence of modulators was set at 100%. Statistically significant enhancement of expression as compared to pathogen-only-exposed DC is indicated (*:p<0.05).

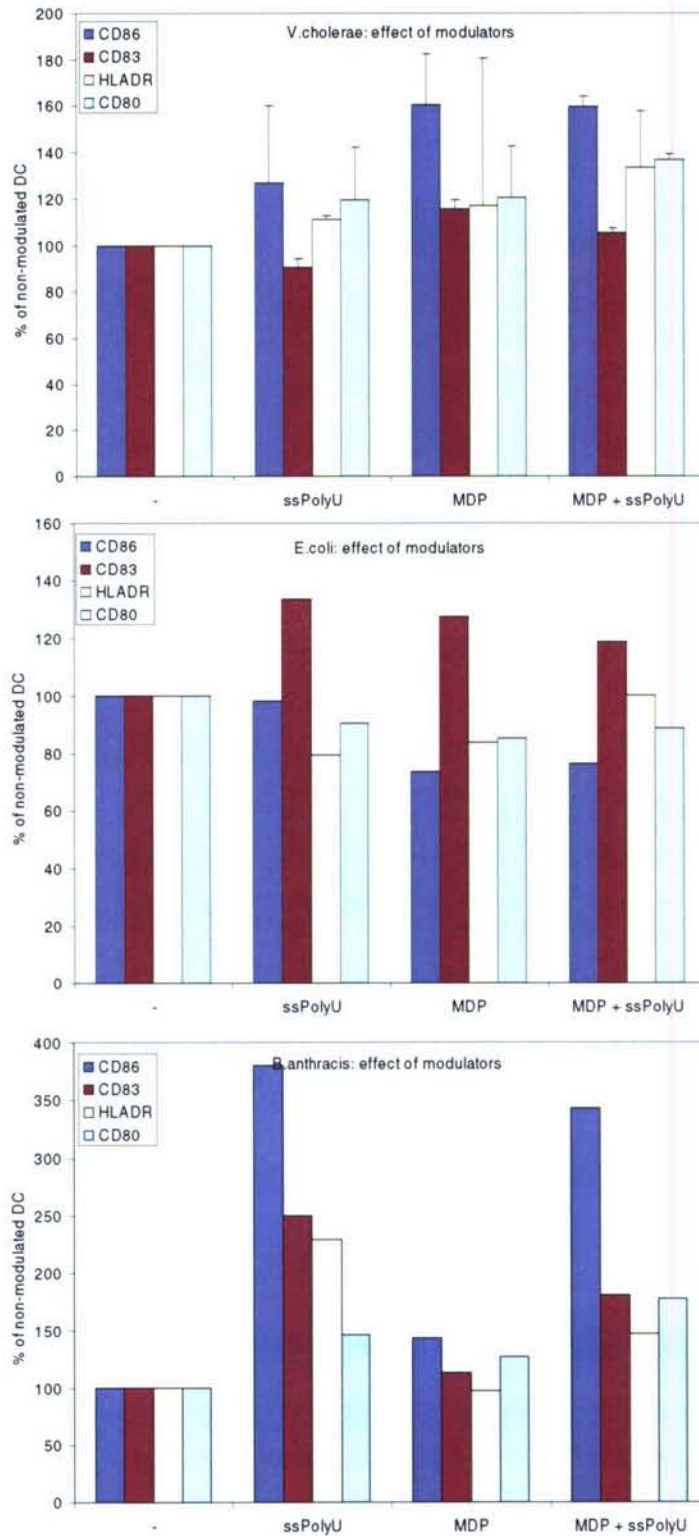


Figure 3B Surface molecules on DC upon stimulation with pathogens in combination with immunomodulators, measured by FACS analysis. For *V.cholerae* the mean+SD of 2 independent experiments is shown, for *E.coli* and *B.anthraxis* a single experiment is shown. The expression on DC exposed to the respective pathogen in the absence of modulators was set at 100%.

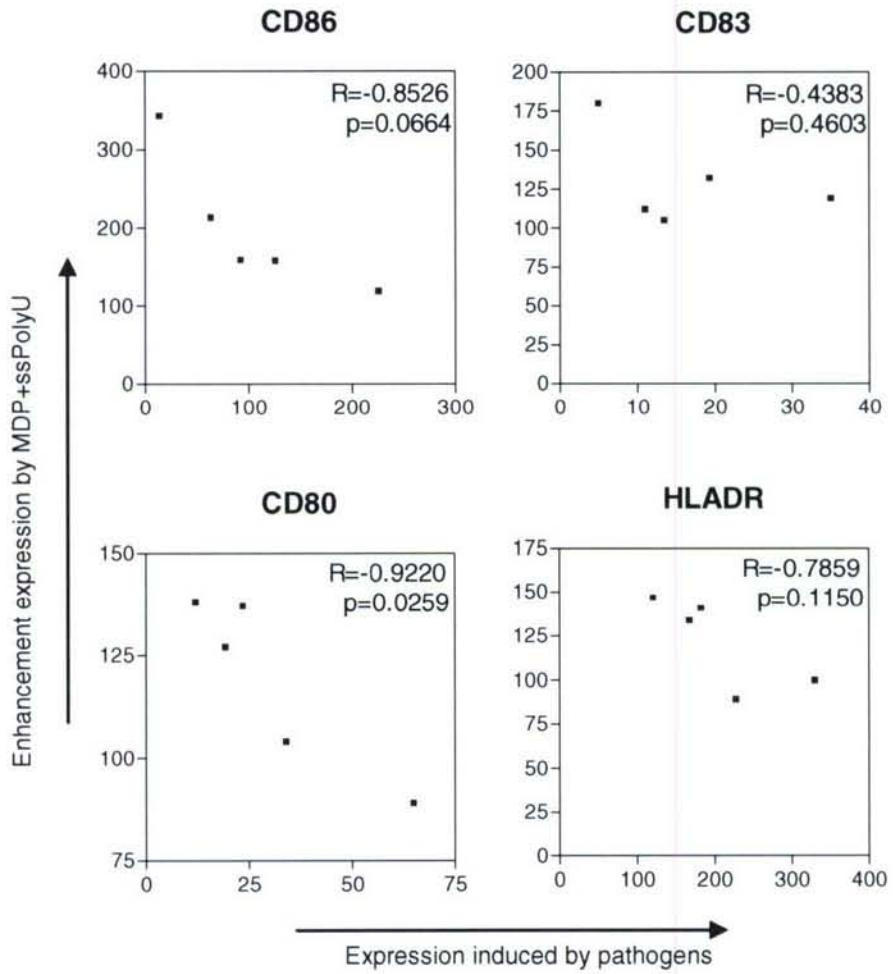


Figure 4 Correlation between the expression level of surface molecules on DC induced by pathogens, and the expression level that is induced by MDP+ssPolyU on top of these pathogen-induced effect. The expression levels were measured by FACS analysis. Expression induced by pathogens are depicted as mean fluorescent intensities, while the extra expression levels upon modulation are given as percentages relative to the effect of the pathogen itself (effect of the pathogen was set at 100%).

3.2 Effects of modulators on T cell polarization

To monitor the effect of the modulators on the T cell polarizing capacity of the DC, DC that were cultured in the presence MPL, MDP, ssPolyU or combinations of these modulators were co-cultured with naïve T cells as described in the Material and Methods section. To analyze T cell polarization 10 days after initiation of the co-culture, T cells were restimulated polyclonally, and interleukin-4 and interferon- γ production were determined as measures for Th2 and Th1 skewing, respectively. As can be seen in Figure 5, none of the modulators enhanced the polarization towards a Th1 response (enhanced IFN- γ , decreased IL-4), which would be the appropriate response to combat intracellular bacteria. It should be noted that DC maturation influences the T cell polarizing capacity; immature DC induce a Th1-polarized profile as compared to neutrally-matured DC (= LPS-matured DC). The polarization profile shown above reflects the differences in maturation of the DC (as shown in Figure 1: MPL and MPL-combinations induce maturation and thus a more neutral T cell profile than immature DC and MDP and ssPolyU-treated DC).

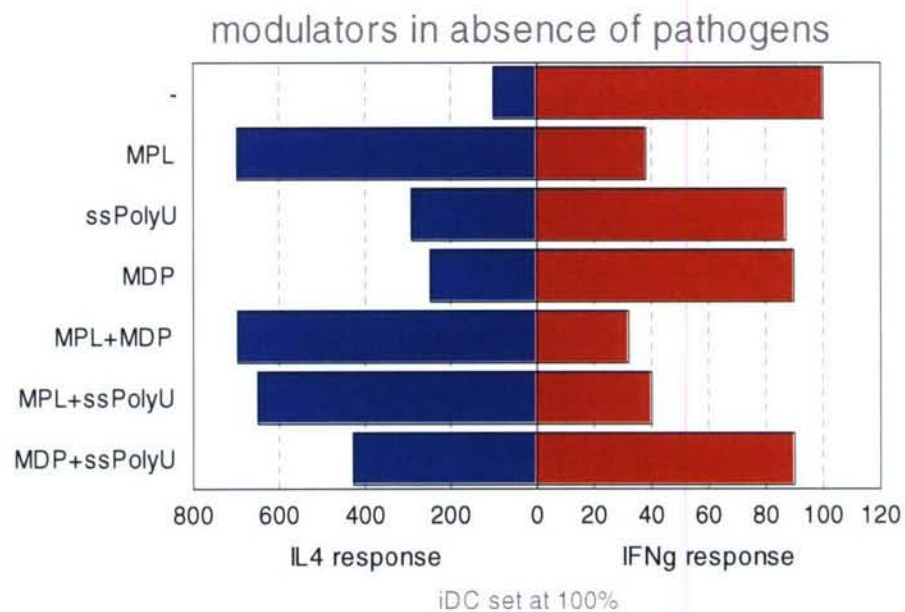


Figure 5 T cell polarization induced by DC that were exposed to (combinations of) modulators, measured by FACS analysis. Percentages of IL-4 producing cells and IFN- γ producing cells in T cells grown in the presence of non-modulated DC (iDC) were set at 100%.

To analyze the effect of the modulators during a bacterial infection, T cell polarization was also analyzed in T cells that were grown in the presence of DC that were exposed to combinations of pathogens and modulators. The results are depicted in Figure 6.

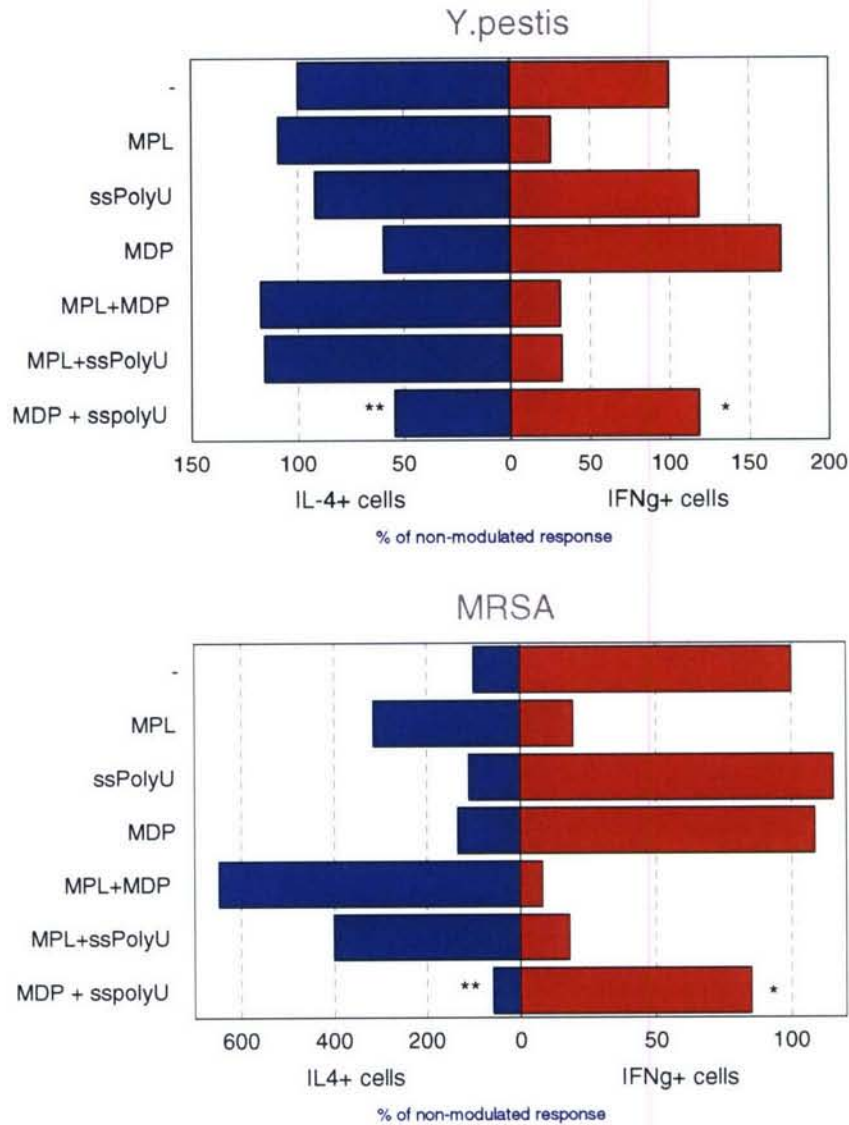


Figure 6A T cell polarization induced by DC that were exposed to *Y.pestis* or MRSA, combined with modulators, measured by FACS analysis. Percentages of IL-4 producing cells and IFN- γ producing cells in T cells grown in the presence of DC that were exposed to pathogens but not modulators were set at 100%. The mean of 3 independent experiments is shown. Statistically significant differences with T cell polarization as compared to the 'pathogen-only' group is indicated (*:p<0.05, **:p<0.01).

In T cell polarization studies, the effects of two pathogens, *Y.pestis* and MRSA, were tested combined with MPL, MDP, ssPolyU and combinations thereof. As shown in Figure 6A, MDP, ssPolyU and the MDP + ssPolyU combination slightly enhanced Th1 polarization, while MPL and MPL-combinations had the opposite effect. T cell polarization followed the same directions in all experiments, although the extend of polarization varied between experiments. Despite these variations, Th1 polarization was significantly enhanced upon modulation with the MDP+ssPolyU combination. Based on the results shown in Figure 6A, the modulators MDP, ssPolyU and the combination of these ligands were tested in combination with 3 additional pathogens, as shown in Figure 6B. In combination with *V.cholerae*, ssPolyU and the MDP+ssPolyU combination again gave rise to significantly enhanced Th1 polarization, however with *E.coli* O157:H7 and *B.anthraxis* this effect was not found (single experiment, no statistics).

Based on the effects of MPL on T cell polarization, MPL was excluded from further studies. The remaining modulators MDP, ssPolyU and the MDP-ssPolyU combination had a modest enhancing effect on DC maturation, and enhanced T cell polarization towards a Th1 direction when combined with some (but not all) pathogens. Therefore, MDP, ssPolyU and their combination will be tested in an *in vivo* experiment, in which mice will be treated with these modulators and subsequently challenged with bacterial pathogens.

A complication with respect to TLR8 and its ligands is that the situation in mice differs from that in humans; TLR8 is expressed on human monocyte-derived DC, but TLR7 is not. However, mice do not express TLR8, but do express TLR7. Moreover, although TLR7 expression in humans is limited to plasmacytoid DC and B cells, TLR7 in mice is widely expressed, resembling the expression distribution of TLR8 in humans. It may well be that TLR7 in mice has a dual function that in humans is covered by TLR7 and TLR8.

In humans, TLR7 and TLR8 each can be activated by unique ligands, but in addition several ligands are known that activate both TLR7 and TLR8. Since ssPolyU activates TLR8 but not TLR7, this modulator cannot be used in a murine infection model. Therefore, a ligand that activates both TLR7 and TLR8 will be used. One of these ligands is CLO75. CLO75 (3M002) is a thiazoloquinolone derivative that stimulates human TLR8, and to a lesser extent human TLR7.

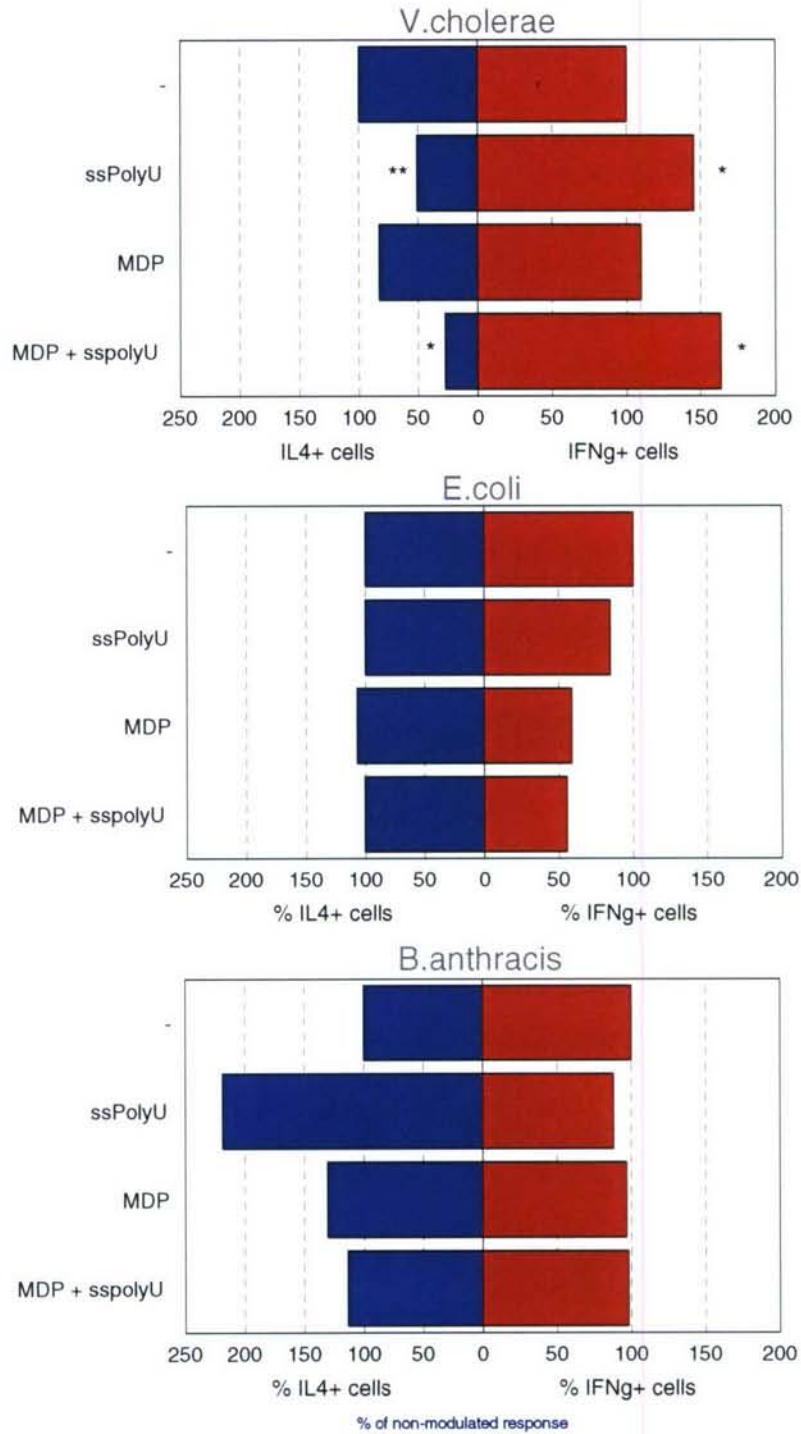


Figure 6B T cell polarization induced by DC that were exposed to *V.cholerae*, *E.coli* O157:H7 or *B.anthraxis*, combined with modulators, measured by FACS analysis. Percentages of IL-4 producing cells and IFN- γ producing cells in T cells grown in the presence of DC that were exposed to pathogens but not modulators were set at 100%. For *V.cholerae* the mean of 2 independent experiments is shown. Statistically significant differences with T cell polarization as compared to the 'pathogen-only' group is indicated (*:p<0.05, **:p<0.01). For *E.coli* and *B.anthraxis* a single experiment is shown.

3.3 Immune activation by the modulators in murine cells

To confirm immune activation by the modulators in mice, cells were isolated from murine spleens, and these cells were stimulated with CLO75, ssPolyU and MDP. Since MDP is reported to be somnogenic and pyrogenic, the use of an analogue that lacks these properties would be preferred. Therefore, murabutide was also included in this experiment. Murabutide (MurNAc-L-Ala-D-GlnOBu) is a safe synthetic immunomodulator derived from MDP that is devoid of pyrogenic activity and lacks somnogenic activity. As a positive control, CpG DNA that activates TLR9 was included in the experiment. More specifically, ODN1826 (5'-tccatgacgttcctgacgtt-3'), a CpG oligodeoxynucleotide that specifically activates murine TLR9 was used.

As shown in Figure 7, the splenocytes were dose-dependently activated by CLO75 and CpG DNA, and not by the other modulators. This is all in agreement with expectations: CLO75 activates both TLR7 and TLR8, while ssPolyU is a TLR8-specific ligand. Murine cells express TLR7 but not TLR8, and can therefore be activated by CLO75 but not by ssPolyU. MDP does not induce cytokine production when used as a stand-alone agent in murine cells [Uehara et al, 2005], and CpG DNA induces IL-6 production via the activation of murine TLR9 [Vollmer et al, 2004].

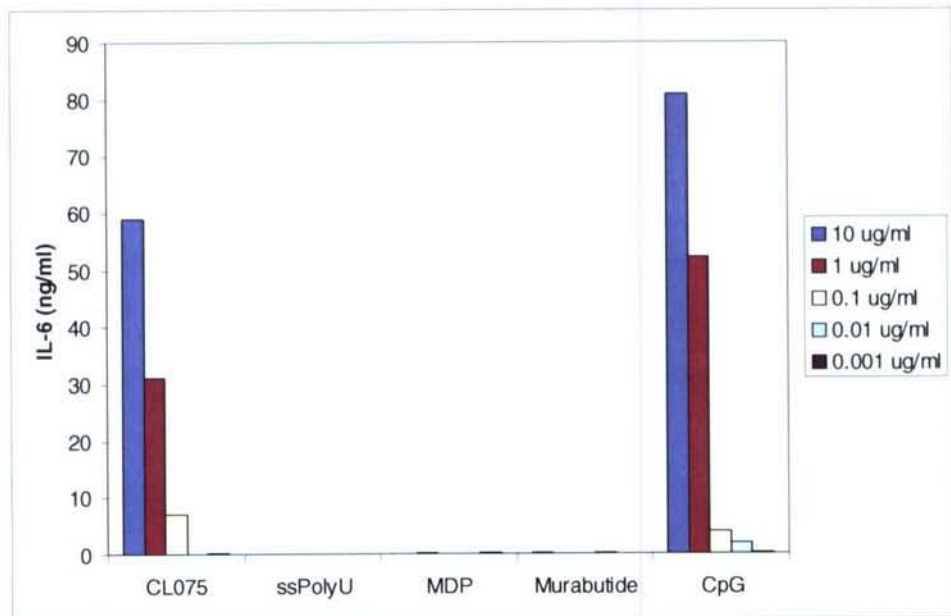


Figure 7 Activation of murine splenocytes by different modulators. IL-6 was measured by ELISA.

To monitor the synergistic effects of MDP and murabutide in the mouse system, splenocytes were stimulated with different concentrations of CLO75 in combination with various concentrations of MDP or murabutide. Figure 8 shows that both MDP and murabutide enhance the IL-6 production that is induced by CLO75. However, MDP shows stronger synergistic effects with CLO75 than murabutide. Therefore, the effects of CLO75 and MDP will be tested in an *in vivo* infection model. As mentioned above, MDP can induce undesired effects. If synergism of CLO75 and MDP can be confirmed *in vivo*, MDP-analogues may be tested for synergistic activity with CLO75 *in vivo*.

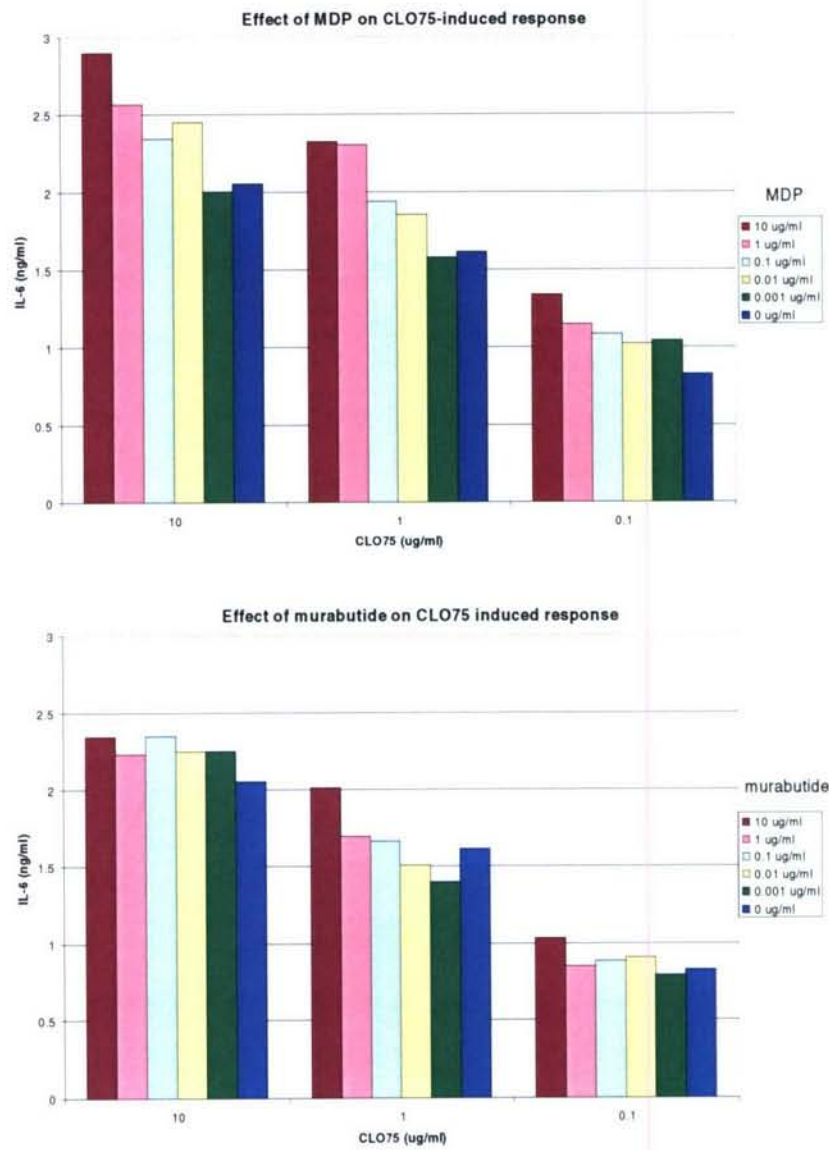


Figure 8 Synergistic effects of MDP or murabutide and CLO75 on induction of IL-6 production in murine splenocytes. IL-6 was measured by ELISA.

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 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 is 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 desirable to administer these vaccines to military personnel, since the possible interactions between different vaccines are unknown. 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 Immunomodulation via dendritic cells

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, termed pattern recognition receptors, which include the Toll-like receptor family [Hemmi et al, 2005] and the NOD-like receptor family [Fritz et al, 2006]. 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 has a special role: it forms the bridge between innate and adaptive immunity. 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 the activation of T cells. The combination of surface molecules on DC and cytokines produced by the DC during contact with the T cells 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.

The central role of the DC in the immune response makes this cell type highly suitable as target for immunomodulation. The effects of pathogens and/or modulators on DC can

be studied *in vitro* in a culture system that was validated in research program V013 (TNO report TNO-DV2 2005-A029). The effects of pathogens in this system are described in TNO report TNO-DV2 2005-A204, the effect of the first immunomodulators tested in this system are described in this report.

To modulate DC, several molecules were selected that activate certain pattern recognition receptors on DC. The mechanisms by which the recognition of microbial molecules by pattern recognition receptors on immune cells leads to host immunity remain poorly defined. Numerous studies in literature now describe the stimulatory effects of molecules that activate DC via a single receptor. However, the thought is now emerging that to induce an effective immune response, microorganisms must stimulate complex sets of pattern recognition receptors such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs). The combined activation of these different receptors can result in complementary, synergistic or antagonistic effects that modulate innate and adaptive immunity. For TLRs, synergistic effects on IL-12 production and Th1 polarization have been described for combinations of TLRs coupled to the adaptor molecule TRIF (TLR3 and TLR4) with TLRs that are located within endosomes (TLR7, 8 and 9) [Napolitani et al, 2005, Fritz et al, 2005].

Although there are quite some studies described in literature now that report the effects of single TLR ligands as vaccine adjuvants or stand-alone immunomodulators in infectious diseases *in vivo*, to our knowledge there are no reports on the effects of combinations of such molecules either as adjuvants or stand-alone agents in *in vivo* infection models. The only evidence of the importance of TLR cooperation *in vivo* comes from studies in which mice defective in single or multiple TLRs are infected with pathogens. For example, mice that have defects in TLR2 and TLR9 are much more susceptible to aerosol *M.tuberculosis* infection than mice that are defective in one TLR [Bafica et al, 2005]. It should be mentioned that for several bacterial and protozoal pathogens, the susceptibility of single-TLR deficient mice to infection is increased under conditions of high-dose challenge. For example, whereas TLR2- or TLR9 defective mice have nearly normal resistance to low-dose challenge with *M.tuberculosis*, at high doses these mice are clearly more susceptible to infection than wild-type mice. This indicates that under high levels of infectious stress, the function of each TLR becomes more crucial for microbial control.

The concept that multiple TLR-ligand interactions are required for the induction of effective host resistance to pathogens has important implications for the design of improved strategies for vaccination and immunotherapy against infectious diseases. Several studies have already convincingly shown the improved efficacy of treatment with multiple TLRs in stimulating cellular immune responses against cancer in *in vivo* animal models. For example, co-administration of polyI:C (TLR3 ligand) and CpG ODN (TLR9 ligand) controls pulmonary metastases in a mouse tumor model more effectively than either of the ligands alone [Whitmore, 2004].

In the studies described in this report, the combined use of TLR4 and TLR8 ligands MPL and ssPolyU, respectively, and the NOD2 (an NLR) ligand MDP was tested with respect to their ability to enhance immunity against bacterial pathogens in the *in vitro* dendritic cell culture system mentioned above. Enhanced immunity in this culture system can be determined either by the induction of enhanced maturation of dendritic cells, or by the induction of enhanced Th1 polarization. The effects of the modulators were determined in the absence and presence of pathogens.

4.3 Effects of modulators on DC maturation

When considering maturation, MPL was the only modulator that was able to induce maturation on its own. When modulators were combined, synergism was found between MDP and MPL, and between MDP and ssPolyU. This is in contrast to findings by Tada et al, who found no synergistic effects of lipid A (TLR4 ligand) and MDP on DC maturation [Tada et al, 2005]. This discrepancy could be explained by the concentration of TLR4 ligand used: in the study of Tada et al, maximal maturation is already induced by lipid A, while we chose our MPL concentration in a way that it induces sub-optimal maturation levels, so that synergism can be detected. Furthermore, MPL and ssPolyU do not synergistically enhance maturation in our hands, which is in agreement with previous findings by [Napolitani et al, 2005], who found that LPS (a TLR4 ligand) and R848 (a TLR7/8 ligand) synergistically enhanced the Th1 polarizing capacity of DC, but not DC maturation. It should be noted that also in the study by Napolitani et al, ligands were used in concentrations that gave maximal maturation levels, so that synergism would be difficult to detect. However, in our set-up where suboptimal concentrations of TLR ligands were used, the TLR ligands still did not synergize to enhance DC maturation.

When DC were exposed to combinations of modulators and pathogens, it was very clear that the effects of the modulators varied when combined with different pathogens. In general, the effects of the modulators on DC maturation were more prominent when the pathogen itself was a weak inducer of DC maturation. Although the effects of the modulators differed when combined with different pathogens, some general observations can be made, namely that MDP did not enhance pathogen-induced maturation while ssPolyU and MPL did enhance pathogen-induced maturation. No evident synergism of the modulators was found when combined with pathogens.

4.4 Effects of modulators on T cell polarization

When considering the T cell polarizing capacity of DC, the modulators ssPolyU and MDP and their combination induced a strong Th1 response when tested in the absence of pathogens, while MPL and the MPL+MDP combination induced a Th2 response. Since the Th1 response is the appropriate response to combat bacterial infections, ssPolyU and MDP seem to be the most suitable modulators. In contrast to published findings [Napolitani et al, 2005; Tada et al, 2005], no synergy of the combined modulators was found on Th1 polarization. In the published studies, the percentage of Th1 cells resulting from modulation with a single ligand was much lower than in our studies. It appears that Th1 polarization in our studies is already maximal and leaves no room for further enhancement by a second modulator. This may be due to the source of the naïve T cells that were used: the donor used for T cell isolation showed already high percentages of IFN- γ producing cells in unmodulated responses. It is possible that synergistic effects are measurable when another T cell donor is used.

When the modulators were tested in combination with MRSA or *Yersinia pestis*, the same trends were found: MDP, ssPolyU and their combination enhanced Th1 polarization (on top of the response induced by the pathogens alone), while MPL alone or combined with other modulators inhibited the Th1 response. ssPolyU, MDP and their combination was further tested in combination with *E.coli*, *V.cholerae* and *B.anthraxis*. In combination with *V.cholerae* the Th1 promoting effects of the modulators was confirmed, however not when the modulators were combined with *E.coli* or *B.anthraxis*. It should be noted that these last two pathogens were tested in only one

experiment. The reason for the failure of the modulators to enhance Th1 polarization is not known.

4.5 Immune activation by the modulators in murine cells

Based on the findings described above, the effects of MDP and ssPolyU and their combination on infection will be studied in a mouse infection model. A complication is that the expression of TLR7 and TLR8 differs between mice and humans: TLR8 is expressed on human monocyte-derived DC, but TLR7 is not. However, mice do not express TLR8, but do express TLR7. Moreover, although TLR7 expression in humans is limited to plasmacytoid DC and B cells, TLR7 in mice is widely expressed, resembling the expression distribution of TLR8 in humans. Thus, TLR7 in mice is present on myeloid DC, and may have the same function as TLR8 in humans. Since ssPolyU activates TLR8 but not TLR7, this modulator cannot be used in a murine infection model. Therefore, CLO75, a ligand that activates both TLR7 and TLR8 will be used in the mouse studies. Immune activity of the modulators that will be used *in vivo* was checked *in vitro*: CLO75 was shown to activate murine splenocytes, and this activation was enhanced when combined with MDP.

4.6 Conclusion

In conclusion, the combination of the NOD2 ligand MDP and the TLR7/8 ligand CLO75 seems to be a promising combination of modulators, which may work to limit infection with pathogens. Although the number of experiments performed is limited and the outcome varies when different pathogens are used, the observed trends support this conclusion. In this respect, one should bear in mind that modulators that are already used clinically as vaccine adjuvants behave in a similar way in this culture system (personal experience D. van der Kleij, data not shown). The effects of this combination of modulators will be evaluated *in vivo* in a murine infection model. The results of the *in vivo* studies will be described in a separate TNO report.

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6 Signature

Rijswijk, July 2007

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Dr P.A.V. van Hooft
Head of Department

TNO Defence, Security and Safety

A handwritten signature in blue ink, featuring a prominent horizontal stroke followed by several loops and a final flourish.

Dr D. van der Kleij
Author

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dr. N. Zegers
ing. L.C.L. Boele
ing. W.E. Kaman-van Zanten
ing. A.M. de Vries
- 32 CDC/BGGZ/CEMG
dr. H. J. Jansen, Projectbegeleider

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

- 4 ex. BS/AL/DMO/SC-DR&D
- 1 ex. DMO/ressort Zeesystemen
- 1 ex. DMO/ressort Landsystemen
- 1 ex. DMO/ressort Luchtsystemen
- 2 ex. BS/AL/CDS/DOBBP/SCOB
- 1 ex. MIVD/AAR/BMT
- 1 ex. TNO Defensie en Veiligheid, Algemeen Directeur, ir. P.A.O.G. Korting
- 1 ex. TNO Defensie en Veiligheid, Directie Directeur Operaties, ir. C. Eberwijn
- 1 ex. TNO Defensie en Veiligheid, Directie Directeur Kennis, prof. dr. P. Werkhoven
- 1 ex. TNO Defensie en Veiligheid, Directie Directeur Markt, G.D. Klein Baltink
- 1 ex. TNO Defensie en Veiligheid, vestiging Den Haag, Manager Waarnemingssystemen (operaties), dr. M.W. Leeuw
- 1 ex. TNO Defensie en Veiligheid, vestiging Den Haag, Manager Informatie en Operaties (operaties), drs. T. de Groot
- 1 ex. TNO Defensie en Veiligheid, vestiging Rijswijk, Manager Bescherming, Munitie en Wapens (operaties), ir. P.J.M. Elands
- 1 ex. TNO Defensie en Veiligheid, vestiging Soesterberg, Manager Human Factors (operaties), drs. H.J. Vink
- 1 ex. Lt kol H. Evertse
BS/AL/CDS/IMS/Afd. Navo-WEU/Stafofficier nucleaire en non-proliferatiezaken
- 1 ex. D.M. van Weel
BS/AL/HDAB
- 1 ex. Drs. E.S.A. Brands
BS/DJZ/IJB
- 1 ex. Lt kol A. Solkesz
HDP/DPB
- 1 ex. Maj R.F.M. Schröder
Las/PBDL/OB