Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco)bacteria


*Department of Immunohematology and Blood Transfusion, Leiden University Medical Centre, Albinusdreef 2, NL-2333-ZA, Leiden, The Netherlands; †DNAX Research Institute, 901 California Avenue, Palo Alto, CA 94304-1104; and ‡Royal Tropical Institute, Meibergdreef 39, NL-1105-AZ, Amsterdam, The Netherlands

Communicated by Johannes van Rood, Europondon Foundation, Leiden, The Netherlands, February 11, 2004 (received for review July 17, 2003)

Macrophages (MΦ) play a central role as effector cells in immunity to intracellular pathogens such as Mycobacterium. Paradoxically, they also provide a habitat for intracellular bacterial survival. This paradoxical role of MΦ remains poorly understood. Here we report that this dual role may emanate from the functional plasticity of MΦ: Whereas MΦ-1 polarized in the presence of granulocyte-MΦ colony-stimulating factor promoted type 1 immunity, MΦ-2 polarized with MΦ colony-stimulating factor subverted type 1 immunity and thus may promote immune escape and chronic infection. Importantly, MΦ-1 secreted high levels of IL-23 (p40/p19) but no IL-12 (p40/p35) after (myco)bacterial activation. In contrast, activated MΦ-2 produced neither IL-23 nor IL-12 but predominantly secreted IL-10. MΦ-1 required IFN-γ as a secondary signal to induce IL-12p35 gene transcription and IL-12 secretion. Activated dendritic cells produced both IL-12 and IL-23, but unlike MΦ-1 they slightly reduced their IL-23 secretion after addition of IFN-γ. Binding, uptake, and outgrowth of a mycobacterial reporter strain was supported by both MΦ subsets, but more efficiently by MΦ-2 than MΦ-1. Whereas MΦ-1 efficiently stimulated type 1 helper cells, MΦ-2 only poorly supported type 1 helper function. Accordingly, activated MΦ-2 but not MΦ-1 down-modulated their antigen-presenting and costimulatory molecules (HLA-DR, CD86, and CD40). These findings indicate that (i) MΦ-1 and MΦ-2 play opposing roles in cellular immunity and (ii) IL-23 rather than IL-12 is the primary type 1 cytokine produced by activated proinflammatory MΦ-1. MΦ heterogeneity thus may be an important determinant of immunity and disease outcome in intracellular bacterial infection.

Mycobacteria can infect human macrophages (MΦ) and cause serious chronic infectious diseases such as tuberculosis and leprosy. MΦ play a crucial role in human host defense by secreting cytokines and chemokines, presenting antigen to T lymphocytes and clearing infectious agents. Type 1 cell-mediated immunity is required for granuloma formation and effective host defense against intracellular pathogens (1), but mycobacteria are able to escape immunity and persist in a nonreplicating state inside MΦ for many years (2). The molecular and cellular mechanisms that underlie the development of effective immunity versus latent infection (or immune escape) and the induction of immunopathology after Mycobacterium tuberculosis infection, however, remain poorly understood.

Mononuclear phagocytes including MΦ are activated through ligation of pattern-recognition receptors such as Toll-like receptors (TLRs) by microbial ligands (3, 4), which is generally considered to potentiate the production of the type 1 cytokine IL-12. IL-12 is a heterodimer of p40 and p35 that drives polarization of naive T cells toward type 1 helper (Th1) cells and induces the release of IFN-γ from Th1 and natural killer cells (5). IFN-γ, in turn, activates MΦ and enhances cytokine secretion, antigen presentation and, supposedly, the bactericidal activity of MΦ (6). The IL-12/IFN-γ axis is critical indeed for the establishment of effective host defense against intracellular pathogens: We and others have reported that human genetic deficiencies in this type 1 cytokine signaling cascade (affecting IL-12p40, IL-12R, IFN-γR, or STAT-1) lead to increased susceptibility to otherwise weakly pathogenic mycobacteria and salmonellae (reviewed in ref. 1).

Dendritic cells (DCs) are highly potent phagocytes that prime naive T cells and control the development of Th1 cells (7, 8). The role of MΦ in Th1-mediated immunity, however, is less clear. It has been reported that MΦ fail to release IL-12 (p40/p35) heterodimer after mycobacterial stimulation (9, 10). Recently, IL-23 was identified as an IL-12-like heterodimer, consisting of IL-12p40 and a novel p19 chain. IL-23, similar to IL-12, induces IFN-γ secretion from T cells (11) and may be involved in type 1 immune defense against mycobacteria (1, 12).

Beside the classical route of MΦ activation, it has become evident in recent years that alternative activation modes of MΦ can be distinguished (for recent reviews see refs. 13 and 14). Whereas classical activation of MΦ by microbial compounds yields a phenotype that is hallmarkmarked by the production of proinflammatory cytokines, alternative activation can lead to an antiinflammatory phenotype, hallmarked by IL-10 as the signature cytokine. Nonclassical antiinflammatory MΦ may also evolve by natural neuroendocrine control mechanisms and play a role in homeostatic processes such as dampening inflammation, scavenging debris, angiogenesis, and wound healing (also tumor outgrowth) (14–16). The characterization of functional human MΦ profiles thus far is incomplete, and the role of MΦ in immunity to mycobacteria remains elusive.

Here we show that highly pure subsets of MΦ with polarized pro- and antiinflammatory phenotypes can be obtained by differentiating human blood monocytes in the presence of the lineage-determining cytokines granulocyte–MΦ colony-stimulating factor (GM-CSF) and MΦ CSF (M-CSF), respectively. Pro- and antiinflammatory MΦ, designated MΦ-1 and MΦ-2, respectively, were characterized for their potential to produce IL-12 and IL-23 to support intracellular mycobacterial growth and present antigens to Th1 lymphocytes. Both MΦ subsets supported the outgrowth of a mycobacterial reporter strain in the absence of any other immune components, but our findings also indicate that MΦ-1 promote whereas MΦ-2 subvert type 1 immunity in the face of (myco)bacterial infection. These results have important implications for our

Abbreviations: MΦ, macrophage(s); TLR, Toll-like receptor; Th1, type 1 helper; DC, dendritic cell; M-CSF, MΦ colony-stimulating factor; GM-CSF, granulocyte–MΦ CSF, moDC, monocyte-derived DC; LPS, lipopolysaccharide.

To whom correspondence should be sent at the present address: Biomedical Primate Research Centre, Department of Parasitology, Lange Kleiweg 157; NL-2288-GJ, Rijswijk, The Netherlands. E-mail: verreck@bpr.nl.

To whom correspondence should be addressed at: Department of Immunohematology and Blood Transfusion, Leiden University Medical Centre, Albinusdreef 2, NL-2333-ZA, Leiden, The Netherlands. E-mail: t.h.m.ottenhoff@lumc.nl.

© 2004 by The National Academy of Sciences of the USA
Materials and Methods

Cells and Microbial Reagents. Monocytes were isolated to high purity by magnetic cell sorting using anti-CD14-coated beads (per manufacturer recommendations, Miltenyi Biotec, Auburn, CA) and subsequently cultured for 6 days in medium (RPMI medium 1640, Gibco/Invitrogen) with 10% FCS (HyClone) and either 50 ng/ml recombinant human GM-CSF (Novartis Pharma, Arnhem, The Netherlands) to generate Mφ-1 or 50 ng/ml recombinant human M-CSF (R & D Systems) to generate Mφ-2. As a control, DCs (monocytes-derived DCs (mo.DCs)) were generated with 1,000 units/ml GM-CSF and 500 units/ml IL-4 as described (17).

Lipopolysaccharide (LPS) from Escherichia coli (serotype 055:B5, Sigma–Aldrich) was used to stimulate Mφ and DCs at 10 ng/ml (unless indicated otherwise). Mycobacterial lysate was obtained by ultrasonication of heat-inactivated Mycobacterium tuberculosis H37Rv, lyophилиzed, and resuspended in PBS as described (18). The lysate was quantified on the basis of bacterial dry weight; cells were resuspended in PBS as described (18). The lysate was quantified by using the cytometric bead assay (BD Biosciences). Cells were stained for 30 min at 4°C by using appropriate coating and rat-anti-hp19 monoclonal antibody 12F12 for detection of HLA-DR2-restricted CD4 T cell clone HA1.7 that is specific for the hemagglutinin (amino acids 306–318) of influenza virus (22). Briefly, antigen-presenting cells were harvested and seeded in triplicates at 2.5 × 10⁶ cells per well in 96-well flat-bottom culture plates in 100 μl in the presence or absence of LPS or mycobacterial sonicate for 4 h. Subsequently, 10² R2F10 cells and recombinant protein or synthetic peptide were added to a final concentration of 10 μg/ml and 100 ng/ml, respectively, in a final volume of 200 μl and incubated for another 72 h. Aliquots of 50 μl of supernatant were harvested and pooled per triplicate to measure IFN-γ secretion (see above), and 0.5 μCi of [³H]thymidine (1 Ci = 37 GBq) was added for another 18 h to measure T cell proliferation.

Cytokine and Chemokine Measurements. IFN-γ secretion was quantified by ELISA (U-CyTech, Utrecht, The Netherlands) with a sensitivity of 20 pg/ml. Specific ELISAs for IL-12p40 and IL-10 were purchased from BioSource International (Camarillo, CA; sensitivity: 20 pg/ml), and IL-12p40/p35 heterodimer was measured by using the cytometric bead assay (BD Biosciences/Pharmingen; sensitivity: 40 pg/ml). IL-23 was measured by ELISA using anti-IL-12p40 monoclonal antibody BP40 (Diaclone) for coating and rat-anti-hp19 monoclonal antibody 12F12 for detection (with a sensitivity of 60 pg/ml).

Polarized Mφ were harvested by using trypsin-EDTA in Hank’s balanced salt solution without Ca/Mg (Gibco/Invitrogen). DCs and Mφ were washed, counted, and seeded in triplicate at 10⁶ cells per 200 μl in 96-well flat-bottom culture plates (Corning Life Sciences) in the presence or absence of stimulating agents as indicated. Supernatants were collected after 24 h (unless indicated otherwise).

Myeloid DCs were stained for 30 min at 4°C by using appropriate coating and rat-anti-hp19 monoclonal antibody 12F12 for detection (with a sensitivity of 60 pg/ml).

Polarized Mφ were harvested by using trypsin-EDTA in Hank’s balanced salt solution without Ca/Mg (Gibco/Invitrogen). DCs and Mφ were washed, counted, and seeded in triplicate at 10⁶ cells per 200 μl in 96-well flat-bottom culture plates (Corning Life Sciences) in the presence or absence of stimulating agents as indicated. Supernatants were collected after 24 h (unless indicated otherwise).

mRNA levels of IL-12p40, IL-12p35, and IL-23p19 were measured by real-time quantitative PCR 8 h after activation. RNA was extracted by using an RNAeasy kit (Qiagen, Valencia, CA) according to manufacturer protocol and reverse-transcribed with oligo(dT)14–18 (Life Technologies) and random hexamer primers (Promega) by using standard protocols. cDNA was analyzed by PCR with a Perkin–Elmer ABI Prism 7700 sequence-detection system. Gene expression was quantified by correcting for the relative expression to 18S RNA levels (19). Mφ and DCs were determined by measuring proliferation and IFN-γ secretion of the HLA-DR2-restricted CD4⁺ T cell clone R2F10 that is specific for the 60-kDa heat-shock protein (amino acids 418–427) of Mycobacterium leprae (21) or the HLA-DR1-restricted CD4⁺ T cell clone HAI.1.7 that is specific for the hemagglutinin (amino acids 306–318) of influenza virus (22). Briefly, antigen-presenting cells were harvested and seeded in triplicates at 2.5 × 10⁶ cells per well in 96-well flat-bottom culture plates in 100 μl in the presence or absence of LPS or mycobacterial sonicate for 4 h. Subsequently, 10² R2F10 cells and recombinant protein or synthetic peptide were added to a final concentration of 10 μg/ml and 100 ng/ml, respectively, in a final volume of 200 μl and incubated for another 72 h. Aliquots of 50 μl of supernatant were harvested and pooled per triplicate to measure IFN-γ secretion (see above), and 0.5 μCi of [³H]thymidine (1 Ci = 37 GBq) was added for another 18 h to measure T cell proliferation.

Results

Polarization of Monocytes into Type 1 and Type 2 Mφ Subsets. Highly pure proinflammatory (IL-12p40⁺) Mφ-1 were obtained after culturing CD14⁺ human blood monocytes for 6 days in the presence of recombinant human GM-CSF; and antiinflammatory (IL-10⁺) Mφ-2 were obtained by using M-CSF. Both procedures yielded CD14⁺CD1a⁻ cells (Fig. 1A) that were predominantly adherent and had an apparent Mφ morphology as judged by microscopy (data not shown). For control purposes, from the same donors CD14⁺CD1a⁺CD83⁺ DCs (mo.DCs) were generated (Fig. 1A). Fig. 1B illustrates that Mφ-1, similar to mo.DCs, secreted high levels of IL-12p40 after activation with either the TLR4-agonist LPS or a sonicate of heat-killed M. tuberculosis that stimulates via TLR2 (23). Although Mφ-1 and Mφ-2 showed similar levels of IFN-γ-induced TLR2 and TLR4 expression (data not shown), activated Mφ-2 did not secrete IL-12p40 (Fig. 1B) but produced high levels of IL-10 in comparison with Mφ-1 or mo.DCs (Fig. 1C). These signature cytokine profiles were found consistently in >10 independent experiments using cells from different donors. Moreover, Mφ-1 but not Mφ-2 produced (high levels of) IL-18, tumor necrosis factor-α, IL-6, and IL-1β (F.A.W.V., unpublished observations).

Regulation of IL-12 and IL-23 Production by Mφ and DCs. Because IL-12p40 can pair with either p35 or p19 to form IL-12 (p40/p35) and IL-23 (p40/p19), respectively, we studied the capacity of Mφ and DCs to produce these cytokines. Fig. 2 shows that Mφ-1 and DCs required exogenous IFN-γ to secrete high levels of IL-12 in response to mycobacteria or LPS. Accordingly, IFN-γ was required to induce strong IL-12p35 gene transcription (Fig. 3B). In the absence of IFN-γ, only LPS-activated mo.DCs but not Mφ-1 produced low levels of IL-12, concordant with the low level of LPS-induced p35 mRNA in these cells (Fig. 3A).

In contrast to IL-12, however, IL-23 was secreted by Mφ-1 as well as mo.DCs in response to both LPS and mycobacteria without requiring exogenous IFN-γ (Fig. 2). These findings supported understanding of Mφ biology in cell-mediated immunity to intra- cellular infections.
by the induction of p40 and p19 mRNA in activated Mφ-1 and mo.DCs (Fig. 3). The highest levels of IL-23 were reproducibly secreted from mo.DCs, Mφ-1 and Mφ-2 highly expressed CD14 but showed no or only weak expression of CD1a or CD83 (the latter after activation by LPS) as determined by flow cytometry. Cytokine secretion was measured up to 72 h after stimulation with M. tuberculosis sonicate (myc) (□), LPS (●), or medium control (○). (B) Whereas activated Mφ-1, similar to DCs, secreted high levels of IL-12p40, Mφ-2 failed to secrete IL-12p40. (C) IL-10, in contrast, was most predominant in Mφ-2. Similar cytokine profiles were obtained with cells from at least 10 independent donors.

The condition of the infected Mφ-1 and Mφ-2, we used a luciferase-transfected M. bovis bacillus Calmette–Guérin reporter strain (bacillus Calmette–Guérin-lux). As reported previously (20, 24), the luciferase activity of bacillus Calmette–Guérin-lux correlated well with bacterial viability as determined by classical colony-forming unit counting (data not shown). In accordance with the higher capacity to bind and/or phagocytose mycobacteria, we found reproducibly enhanced outgrowth of mycobacteria at day 6 after infection in Mφ-2 over Mφ-1 over a range of 20–2.5 infecting mycobacteria per host cell (Fig. 4B). The condition of the infected Mφ-1 and Mφ-2 as judged by light microscopy revealed no significant differences during the incubation period. This was corroborated further by calcine viability staining of the host cells [Mφ-1: 31,977 (±3,449) cps; Mφ-2: 28,936 (±3,656) cps (n = 3)]. Notably, the predominant cytokines in the supernatants of Mφ-1 and Mφ-2 at the end of the infection period remained IL-12p40 and IL-10, respectively (data not shown), suggesting that Mφ-1 and Mφ-2 represent stable subsets in the Mφ spectrum.

Mφ-1 but Not Mφ-2 Support Th1 Function After Mycobacterial Stimulation. To address the antigen-presenting capacity of Mφ-1 and Mφ-2, we used an HLA-DR2-restricted Th1 reporter clone (R2F10) that recognizes a M. leprae–specific HSP60 epitope that is lacking from M. tuberculosis. Fig. 5A shows that Mφ-1 and mo.DCs both were effective in inducing proliferation of R2F10 when presenting M. leprae HSP60 protein or peptide antigen. Proliferation of R2F10 Th1 cells induced by Mφ-1 was not significantly affected by preactivation of Mφ-1 with M. tuberculosis sonicate before adding specific antigen (Fig. 5A). In contrast to Mφ-1, however, Mφ-2 stimulated R2F10 relatively poorly, and both pro-
protein- and peptide-mediated T cell activation were reduced further when Mφ-2 had been preactivated by mycobacterial stimulation (Fig. 5A). Similar poor antigen presentation by Mφ-2 was observed when LPS was used to preactivate the cells (Fig. 5B), indicating that the subversion of Th1 cell activation by Mφ-2 was not unique to M. tuberculosis activation. Similar results were obtained with the HLA-DRI-restricted, influenza hemagglutinin-specific T cell clone HA1.7, as illustrated by the dose-dependent reduction in the proliferation of these cells induced by Mφ-2 that had been preactivated with increasing doses of M. tuberculosis sonicate (Fig. 5C). Fig. 5D shows that (preactivated) Mφ-1, albeit less efficiently than mo.DCs, supported antigen-specific IFN-γ secretion by R2F10 cells. In contrast, IFN-γ secretion was poorly supported by Mφ-2, and preactivation further reduced this ability (Fig. 5D). In accordance with their poor antigen-presenting capacity, activated Mφ-2 but not Mφ-1 or mo.DCs down-regulated the expression of HLA-DR and CD86 (but not CD80; Fig. 6) as well as CD40 (data not shown).

Thus, although Mφ-1 (and mo.DCs) promote activation of Th1 cells, Mφ-2 fail to promote Th1-mediated immunity efficiently after activation by microbial components.

Discussion

Mφ are the major population of tissue-resident mononuclear phagocytes and the predominant targets for infection by intracellular pathogens including mycobacteria. Mφ play a dual role in antimycobacterial host defense that currently is poorly understood: They contribute to cell-mediated immunity and bacterial elimination but also provide an essential niche for intracellular bacterial survival and escape from host defense mechanisms. Here we identify two distinct human Mφ subsets, Mφ-1 and Mφ-2, that display largely opposite functions. Although both Mφ populations can be infected and support the outgrowth of mycobacteria (in the absence of any other immune components), Mφ-1 promote type 1 cellular immunity, whereas Mφ-2 are poor antigen-presenting cells for supporting type 1 immunity. Mycobacterium-activated Mφ-1 secrete IL-23 (p40/p35), whereas activated Mφ-2 fail to produce IL-23 or IL-12 and predominantly secrete IL-10.

It is well established that type 1 cell-mediated immunity is essential for optimal host defense against intracellular pathogens (1, 6, 25), but it is unresolved how (different functional) Mφ (subsets) contribute to type 1 immunity in antimycobacterial host defense. In the present study, we generated proinflammatory Mφ-1 using the lineage-determining cytokine GM-CSF, which is associated with inflammation. These Mφ-1 efficiently supported the antigen-specific function of Th1 cells. Importantly, our findings indicate that these Mφ-1 initially secrete IL-23 (p40/p19) but no IL-12 (p40/p35) after mycobacterial stimulation. Similar to that for monocytes and mo.DCs (26–28), enhancement of IL-12 production after microbial stimulation of Mφ-1 required IFN-γ, which activates transcription of the IL-12p35 gene. This regulation of IL-12 production may reflect an important function of IFN-γ in enhancing type 1 cellular immunity against intracellular pathogens. Although activation of both DCs and Mφ-1 in the presence of IFN-γ reduced the induction of IL-23-specific p19 mRNA, only DCs reduced IL-23 protein levels under these conditions. Mφ-1, in contrast, enhanced IL-23 secretion after (myco)bacterial stimulation in the presence of IFN-γ. Additional studies are required to unravel the (posttranscriptional) mechanism that is responsible for this differential regulation of IL-23 secretion between mo.DCs and Mφ-1. Although we confirm previous studies that were unable to detect IL-12 heterodimer (9, 10), our study now demonstrates that mycobacterial stimulation of human proinflammatory Mφ triggers IL-23 secretion.
cells to produce GM-CSF (33). Thus, by enhancing GM-CSF production, IL-23 may drive differentiation of newly recruited monocytes to a proinflammatory Mφ phenotype.

Over the last few years it has become clear that Mφ are highly heterogeneous, and nonclassical antiinflammatory subsets have been identified (13, 14). Our results show that human Mφ polarized by M-CSF stably display such a nonclassical phenotype, secreting IL-10 but no IL-12(p40) in response to (myco)bacteria. The lack of type 1 cytokine secretion, the high production of IL-10, and the profound down-regulation of HLA, CD86, and CD40 by activated Mφ-2 are likely to contribute to their poor Th1-activating capacity (34–37). Mφ-2 expressed similar levels of TLR4 and TLR2 as Mφ-1, suggesting that the functional differentiation between these two subsets emanates from differential signaling or gene-expression profiles rather than from divergent patterns of innate immune recognition. The enhanced binding and uptake of mycobacteria by Mφ-2 compared with Mφ-1 (already at 0°C) suggests differential expression of a cell surface receptor and also fits with the notion that nonclassical Mφ display enhanced endocytosis (15). This may account for the elevated outgrowth of M. bovis bacillus Calmette–Guerin in Mφ-2.

Gordon and coworkers (13, 38) have described alternatively activated Mφ after treatment with IL-4 or IL-13, which in contrast to Mφ-2 produce IL-10 without microbial stimulation. Also unlike alternatively activated Mφ, activated Mφ-2 failed to release Mφ-derived chemokine (MDC/CCL22) or thymus- and activation-regulated chemokine (TARC/CCL17) (unpublished observations). However, activated Mφ-2 readily produced other chemokines (e.g., MCP-1/CCL2, IP-10/CXCL10, and MIP-1β/CCL4; unpublished data), suggesting that Mφ-2 can attract and regulate other immune cells such as monocytes and lymphocytes. The term “type 2 Mφ” has been used to describe IL-12–IL-10+ Mφ in the mouse, obtained by stimulation through TLR, CD40, or CD44 ligation in the presence of Fcγ-receptors ligating immune complexes (39). Additional analyses should reveal how these type 2 Mφ relate to the human Mφ-2 in this study.

A murine Leishmania infection model has indicated that IL-10 plays a central role in the maintenance of latent infection by intracellular pathogens (40). Moreover, IL-10-deficient mice display increased antimycobacterial immunity with concordant higher levels of tumor necrosis factor-α and lower bacterial burden (41). Therefore, IL-10 produced by activated Mφ-2 may inhibit optimal host defense and promote latent infection and immune escape by mycobacteria. M-CSF used to generate Mφ-2 is a ubiquitous serum protein, which may indicate that under normal homeostatic conditions Mφ are switched to an antiinflammatory mode. M-CSF-treated Mφ have been described to induce T cell hyporesponsiveness in an indoleamine 2,3-dehydrogenase-dependent fashion and have been implicated in the maintenance of peripheral tolerance (42–44). Furthermore, MCP-1, which is strongly secreted by Mφ-2.
whereas Mγ-1 promotes cell-mediated immunity, Mγ-2 seems to down-regulate type 1 cell-mediated immunity by various mechanisms and may promote chronic mycobacterial infection.

Intracellular pathogens generally interact with and modulate their host cells, and \( M. tuberculosis \) has an intrinsic capacity to interfere with the classical IFN-γ and TLR-mediated activation pathways of Mγ (48, 49). Our findings imply that not only active interference of pathogenic with Mγ signaling pathways but also the plasticity in the human Mγ compartment may critically affect host defense against intracellular infections. Beside providing a rationale for the paradoxical role of Mγ in combating intracellular pathogens while also providing a niche that is sequestered from immunosurveillance, our study also provides a model for additional investigation of Mγ plasticity and Mγ-derived IL-23 during inflammation, protective immunity, and immunopathology in mycobacterial infections and immunological diseases in general.

We thank Drs. F. Konig and D. Roelen for critically reading the manuscript. Our work was supported by the Netherlands Leprosy Foundation, the Netherlands Organization for Scientific Research, the Commission of the European Community, and the Royal Netherlands Academy of Arts and Sciences.