Cell Multiplication

DOI: 10.1002/anie.200604651

Multiplication of Human Natural Killer Cells by Nanosized Phosphonate-Capped Dendrimers**

Laurent Griffe, Mary Poupot, Patrice Marchand, Alexandrine Maraval, Cédric-Olivier Turrin, Olivier Rolland, Pascal Métivier, Gérard Bacquet, Jean-Jacques Fournié, Anne-Marie Caminade, Rémy Poupot,* and Jean-Pierre Majoral**

Dedicated to Professor Jean-Jacques Bonnet

Nanotechnology appears as the main innovative breakthrough in the last decade for many scientific fields and has in particular induced the emergence of "nanomedicine".[1] This rapidly expanding multidisciplinary field greatly benefits from chemistry, with the elaboration of highly sophisticated nanoscale and multifunctional devices.[2] Dendrimers, in particular, which are precisely defined and tunable hyperbranched synthetic polymers with layered architectures,[3] show promise in several biomedical applications.[4] Indeed, as a result of their size range (a few nanometers) and their high structural and chemical homogeneity, they are suitable for manipulations at the molecular level. Furthermore, their multivalency owing to the presence of a large number of terminal units may induce multiple simultaneous interactions with biological entities that should be quantitatively and qualitatively different from the individual interactions displayed by monomeric constituents (the "cluster glycoside effect"[5]). One important field of research using dendrimers in nanomedicine concerns oncology. Indeed, dendrimers can be used as drug carriers that are able to enhance aqueous solubility, improve drug transit across biological barriers, and target the drug to diseased tissues.[4]

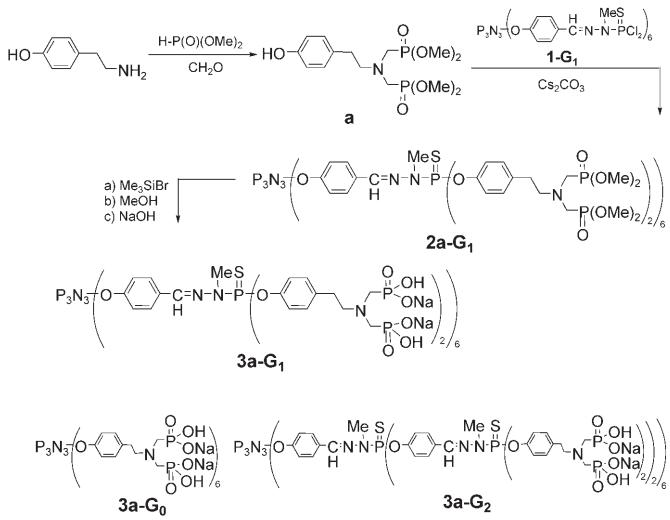
Besides the use of specific anticancer drugs, it is known that the immune system is able to fight against cancers; the challenge is to "manipulate" it to increase its efficiency.[6] Peripheral blood immune cells are present within the circulating pool of blood, easily accessible, and widespread in the whole body, and thus they are a target of choice for such purposes. The immune system in blood comprises several kinds of cells derived from stem cells in bone marrow, in particular natural killer (NK) cells, monocytes and dendritic cells, which are part of the innate immunity, and B and T lymphocytes which are part of the adaptive immunity (they require a prior sensitization). Increasing artificially the number of blood immune cells generally necessitates complex and poorly available biological molecules/entities. However, it has been shown previously that small molecules such as pyrophosphates (referred to as phosphoantigens)[7] and amino-bisphosphonates[8] can activate and/or multiply the TCR$\gamma\delta^+$ subset of T lymphocytes, a group of T lymphocytes at the borderline between adaptive and innate immunity.[9] Thus, it appeared interesting to graft phosphorus derivatives and especially phosphonates, which are more stable than phosphates and insensitive to phospholipases[10] and phosphatases,[11] as end groups of dendrimers and to test their behavior towards human immune blood cells.

Up to now, very few dendrimers with phosphonate (phosphonic acid salts) end groups have been synthesized and characterized.[12,13] Here, we report several methods of synthesizing dendrimers capped with phosphonate end groups. The backbone of the selected dendrimers also contains phosphorus as branching points,[14] and the biocompatibility of this backbone was previously demonstrated.[15] The first series of phosphonate-capped dendrimers was synthesized by nucleophilic substitution of the chlorine atoms of dendrimer **1-G$_n$** ($n = 0$–2)[16] by a phenol (**a**) derived from tyramine bearing two dimethylphosphonate groups, in the presence of cesium carbonate (Scheme 1). The azabisdimethylphosphonate end groups of dendrimers **2a-G$_n$** were then treated with trimethylsilyl bromide, methanol, and finally with sodium hydroxide to afford dendrimers **3a-G$_n$**. Sodium salt derivatives were needed to ensure their solubility in water (dendrimers with phosphonic acid end groups are insoluble). Generations zero (six azabisphosphonate end groups) to two (24 azabisphosphonate end groups) were

[*] Dr. L. Griffe,[+] Dr. P. Marchand, Dr. A. Maraval, Dr. C.-O. Turrin, O. Rolland, Dr. A.-M. Caminade, Dr. J.-P. Majoral
Laboratoire de Chimie de Coordination, CNRS
205 route de Narbonne, 31077 Toulouse Cedex 4 (France)
Fax: (+33) 5-6155-3003
E-mail: caminade@lcc-toulouse.fr
majoral@lcc-toulouse.fr
Dr. M. Poupot,[+] Dr. J.-J. Fournié, Dr. R. Poupot
INSERM U.563
Centre de Physiopathologie de Toulouse-Purpan
Hôpital Purpan, 31300 Toulouse (France)
Fax: (+33) 5-6274-4548
E-mail: remy.poupot@toulouse.inserm.fr
Dr. P. Métivier, Dr. G. Bacquet
Centre de Recherches d'Aubervilliers, Rhodia
52 rue de la Haie Coq, 93308 Aubervilliers Cedex (France)
[+] Contributed equally to this work.

[**] We thank Rhodia (grants to L.G., P.M., and O.R.), the CNRS (grant to A.M.), the EC (F.S.E. grant to M.P.), and the Région Midi-Pyrénées ("Biotherapy" program) for financial support. We also thank Dr. Yannick Coppel for NMR spectra.

Supporting information for this article (including syntheses, characterizations, copy of ^{1}H, ^{13}C, and ^{31}P NMR spectra of all compounds, materials and methods for biology, and flow cytometry figures) is available on the WWW under http://www.angewandte.org or from the author.

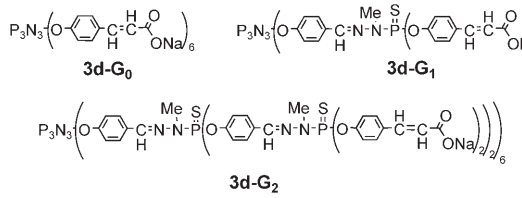


Scheme 1. Synthesis of first-generation dendrimer **3a-G$_1$**, and chemical structures of zero- and second-generation dendrimers **3a-G$_0$** and **3a-G$_2$**, respectively.

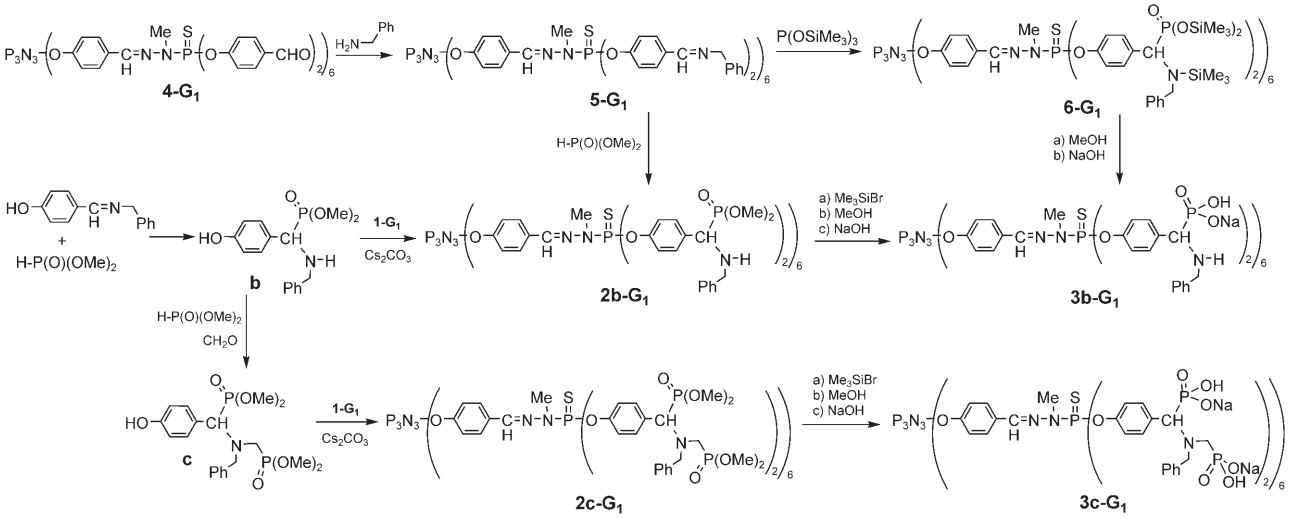
synthesized to determine the influence of the loading in phosphonate groups on the biological properties.

We also varied the surface of these dendrimers to determine the influence of the type of end groups. We chose in particular to graft an unsymmetrical bisphosphonate (to see the influence of the local geometry), a monophosphonate (to see the difference between monodentate and bidentate end groups), and a carboxylate (to see if phosphonates are needed or only negative charges). First, we synthesized two phenols bearing either one (**b**) or two (**c**) dimethylphosphonates which were obtained by hydrophosphinylation of an imine with dimethylphosphite, followed by a Kabachnik–Fields reaction with dimethylphosphite and formaldehyde (Scheme 2). The grafting of these phenols to the first-generation dendrimer **1-G$_1$** affords dendrimers **2b-G$_1$** (aza-monodimethylphosphonate end groups) and **2c-G$_1$** (unsymmetrical azabisdimethylphosphonate end groups). In the case of the monodimethylphosphonate, we observed in some experiments a small percentage of coupling through the NH bond, thus we designed another way to synthesize **2b-G$_1$** consisting of a condensation reaction of the aldehyde end groups of **4-G$_1$**[16] with benzylamine to afford **5-G$_1$**. The resulting imine bonds were then submitted to addition reactions with dimethylphosphite to afford cleanly **2b-G$_1$**. Dendrimers **2b-G$_1$** and **2c-G$_1$** were then converted into the corresponding water-soluble phosphonic acid salts **3b-G$_1$** and **3c-G$_1$**, respectively, by using the method already described in Scheme 1. Note that **3b-G$_1$** is also obtainable from **5-G$_1$** by addition of $P(OSiMe)_3$ to the imines to afford **6-G$_1$**, followed by reaction with MeOH and NaOH (Scheme 2).

The series of dendrimers **3d-G$_n$** ($n = 0$–2)[17] with another type of negatively charged end groups (carboxylates) was also synthesized by carrying out a modified Doebner reaction on dendrimers **4-G$_n$**.



In a first attempt, the first generation of the symmetrical azabisphosphonate dendrimers **3a-G$_1$** was added to cultures of human peripheral blood mononuclear cells (PBMCs; white blood cells issued from healthy donors) supplemented with interleukin-2 (IL-2). We recently reported that such dendrimers are able to activate monocytes within 30 minutes but not to multiply them.[18] To our great surprise, a very different behavior was observed for longer times of culture. Indeed, an important increase in the number of PBMCs was observed (proliferation index 5.5 in two-week-old cultures with 20 μM **3a-G$_1$**) and phenotyping of the cells multiplied in cultures with **3a-G$_1$** revealed the prominence of NK cells (with some T cells). Interestingly, these NK cells express a high level of the membranous marker CD56 and a low level of the membranous marker CD16, thus indicating that they are proliferative NK cells[19] (see the Supporting Information). Experiments with PBMCs obtained from six healthy donors revealed in all cases an important increase in both the percentage and the



Scheme 2. Synthesis of dendrimers with monophosphonate (**3b-G$_1$**) or unsymmetrical bisphosphonate (**3c-G$_1$**) end groups.

number of NK cells when the results were compared with IL-2 alone and with IL-2 and **3a-G$_1$** (Figure 1). After four weeks in culture, a mean multiplication of the number of NK cells by a factor of 105 was achieved in medium supplemented with IL-2 and **3a-G$_1$** versus a mean multiplication only by a factor of 7.5 in medium supplemented with IL-2 alone. These large-scale prototype cultures started with PBMCs comprising 1 million NK cells on average; multiplications over 500-fold were obtained with some donors (Figure 1).

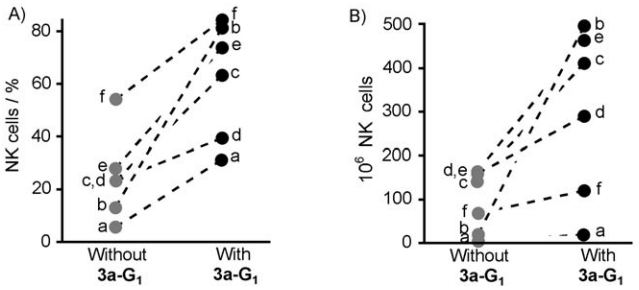


Figure 1. A) Number and B) percentage of NK cells from four-week-old cultures with IL-2 alone (gray dots) or with IL-2 and 20 μM **3a-G$_1$** dendrimer (black dots). Data points a–f represent six healthy donors.

Human NK cells constitute a key population of innate immunity implicated in early immune responses against viral,[20] bacterial,[21] and parasitic infections,[22] and they play a crucial role in anticancer immunity.[23] However, the proliferation of NK cells is extremely tedious to achieve[24] and there are still no means of producing large batches of NK cells necessary for the development of NK-cell-based therapies. These results prompted us to verify if the NK cells generated in cultures with **3a-G$_1$** are fully functional and mature. For this purpose, their ability relative to uncultured NK cells to kill the same cancer cell lines was successfully tested and found to show the same efficiency (15 cell lines tested, seven different leukemia and eight different carcinoma; see Figure 2). Furthermore and most importantly, autologous lymphocytes were preserved (no aggressiveness of the NK cells generated with dendrimers against lymphocytes of the same donor).

These very important results encouraged us to determine the main determinants in the properties of **3a-G$_1$**. First, we checked the dose/effect relationship, which indicated that **3a-G$_1$** is the most active at 20 μM (Figure 3A). Then, we established low-scale routine cultures (i.e. three-week cultures starting with 1×10^5 NK cells on average) to screen the bioactivity of the variety of dendrimers described above. This culture procedure was applied to determine if the activity is only linked to the concentration of bisphosphonates in the culture medium. Figure 3B demonstrates that the generation has a greater influence than the total concentration of bisphosphonates, with **3a-G$_1$** being the most active of the **3a-G$_n$** series.

Comparison of the **3a-G$_n$** series with the **3d-G$_n$** series (n = 0–2) clearly indicated that azabisphosphonate end groups rather than carboxylate end groups afford the highest multiplication of NK cells. Furthermore, symmetrical azabisphosphonate groups gave better results concerning the number of NK cells but similar results concerning the

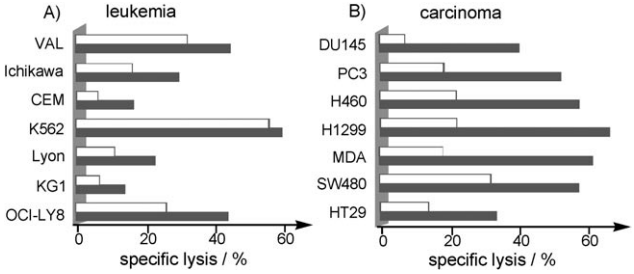


Figure 2. Specific lysis (destruction) of various leukemia (A) and carcinoma (B) cells by NK cells generated in cultures with dendrimer **3a-G$_1$**. Two ratios of NK/cancerous cells were used (white bars: 3:1; gray bars: 10:1).

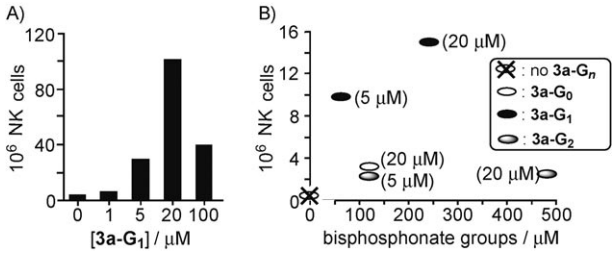


Figure 3. A) Dose/effect titration for **3a-G$_1$** used in large-scale cultures (20 mL). B) Low-scale cultures (5 mL) with various concentrations (in brackets) of **3a-G$_n$** (n = 0–2) or without dendrimer. The x axis is the concentration of end groups (bisphosphonates) obtained by multiplying the molar concentration of dendrimer by 6 for **3a-G$_0$**, by 12 for **3a-G$_1$**, and by 24 for **3a-G$_2$**.

percentage of NK cells than unsymmetrical azabisphosphonate (**3c-G$_1$**) or azamonophosphonate (**3b-G$_1$**) groups when grafted on first-generation dendrimers (Figure 4). Thus, besides the generation, the geometry of the end groups plays an important role also.

In conclusion, we have demonstrated that phosphonates grafted to the surface of phosphorus-containing dendrimers display the unexpected property of dramatically and selectively promoting the multiplication of human natural killer cells. Depending on the size of the dendrimers and on the type, number, and even the geometry of the end groups they bear, large differences in their bioactivity toward NK cell multiplication are observed. In view of the virtually unlimited number of chemical structures of dendrimers, a more detailed, quantitative structure–activity relationship study is needed. However, the multiplication observed of up to 500-fold in certain cases is unprecedented. Furthermore, the

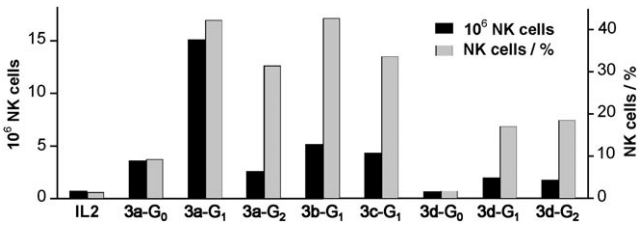


Figure 4. Numbers (black bars) and percentages (gray bars) of NK cells from three-week-old cultures with IL-2 alone or with IL-2 and 20 μM **3x-G$_n$** (x = a–d; n = 0–2) dendrimers. All experiments were conducted on samples of blood issued from the same donor.

bioactivity of the NK cells generated in the presence of dendrimers is not modified, in contrast to what was previously observed with known ligands activating NK cell receptors.[25] Cultures with these dendrimers did not induce activation or inhibition of the NK cells lytic response nor compromise direct toxicity for their target cells and preserve autologous lymphocytes. Thus, these dendrimers constitute a new tool in "nanomedicine". Taking into account the known properties of NK cells, the straightforward production of therapeutic-scale batches of functional NK cells might accelerate the advent of NK cell therapies against infectious and malignant diseases.

Experimental Section

See the Supporting Information for full experimental data, including copies of ^{1}H, ^{13}C, and ^{31}P NMR spectra and details of materials and exhaustive methods for the biology part. The synthesis of dendrimer **3a-G$_1$** is shown here as an example.

Synthesis of phenol **a**: A solution of formaldehyde (37% in water, 8 mL, 104 mmol) was added to a solution of tyramine (7 g, 51.2 mmol) in THF (50 mL). The resulting mixture was stirred for 30 minutes at room temperature, and then dimethylphosphite (10 mL, 110 mmol) was added. The mixture was stirred at room temperature for 24 h. Brine (50 mL) was added to the crude material, which was then extracted with ethyl acetate (3×200 mL). The combined organic phases were dried over $MgSO_4$ and concentrated under reduced pressure. The resulting crude oil was purified by column chromatography (silica, acetone, $R_f = 0.25$) to afford phenol **a** as a colorless oil (65% yield).

Synthesis of dendrimer **2a-G$_1$**: Cesium carbonate (428 mg, 1.314 mmol) and phenol **a** (501 mg, 1.314 mmol) were added to a solution of dendrimer **1-G$_1$** (182 mg, 9.95×10^{-2} mmol) in acetone (5 mL). The reaction mixture was stirred at room temperature overnight then centrifuged, and the resulting clear solution was concentrated under reduced pressure. The residual oil was eluted on a plug of silica with acetone to remove the unreacted phenol with acetone/methanol (1:1) or acetone/water (7:3). The resulting solution of dendrimer was concentrated to dryness under reduced pressure, the residue was dissolved in CH_2Cl_2 (10 mL), and the solution was dried over Na_2SO_4, filtered (micropore, 0.2 μm), and finally concentrated under reduced pressure to afford dendrimer **2a-G$_1$** as a colorless oil (85% yield).

Synthesis of dendrimer **3a-G$_1$**: Trimethylsilyl bromide (375 μL, 2.84 mmol) was added dropwise to a solution of dendrimer **2a-G$_1$** (300 mg, 5.03×10^{-2} mmol) in acetonitrile (15 mL) maintained at 0 °C. The reaction mixture was stirred at room temperature overnight, then the solvent was evaporated to dryness under reduced pressure. The crude residue was washed with methanol (2×5 mL) for one hour at room temperature and dried under reduced pressure. The resulting white solid was washed once with diethyl ether (20 mL) and then transformed into its sodium salt by adding one equivalent of sodium hydroxide per terminal phosphonic acid to a suspension of the dendrimer in water (1 mL/100 mg). The resulting solution was lyophilized to afford dendrimer **3a-G$_1$** as a white powder (80% yield).

Biological procedures: Fresh blood samples were collected from healthy adult donors, and PBMCs were prepared on a Ficoll-Paque gradient by centrifugation (800 g, 30 min at RT). For NK cell multiplication, PBMCs were cultured in complete RMPI 1640 medium supplemented with penicillin and streptomycin (both at 100 U mL^{-1}), 1 mM sodium pyruvate, 10% heat-inactivated fetal calf serum, and recombinant human IL-2 (400 U mL^{-1}). Cultures were started with 1.5×10^{6} cells mL^{-1}, and this density was maintained along the culture by adding fresh medium. Sterile filtered solutions of the specified dendrimers were added at a final concentration of 20 μM.

Received: November 15, 2006
Published online: February 15, 2007

Keywords: biological activity · dendrimers · immunology · NK cells · phosphonates

[1] V. Wagner, A. Dullaart, A. K. Bock, A. Zweck, *Nat. Biotechnol.* **2006**, *24*, 1211 – 1217.
[2] R. Haag, F. Kratz, *Angew. Chem.* **2006**, *118*, 1218 – 1237; *Angew. Chem. Int. Ed.* **2006**, *45*, 1198 – 1215.
[3] a) G. R. Newkome, C. N. Moorefield, F. Vögtle, *Dendrimers and Dendrons: Concepts, Syntheses, Applications*, Wiley-VCH, Weinheim, **2001**; b) J. M. J. Fréchet, D. A. Tomalia, *Dendrimers and Other Dendritic Polymers*, Wiley, Chichester, **2001**.
[4] C. C. Lee, J. A. MacKay, J. M. J. Fréchet, F. C. Szoka, *Nat. Biotechnol.* **2005**, *23*, 1517 – 1526.
[5] J. J Lundquist, E. J. Toone, *Chem. Rev.* **2002**, *102*, 555 – 578.
[6] O. J. Finn, *Nat. Rev. Immunol.* **2003**, *3*, 630 – 641.
[7] M. Poupot, J. J. Fournié, *Immunol. Lett.* **2004**, *95*, 129 – 138.
[8] V. Kunzmann, E. Bauer, J. Feurle, F. Weissinger, H. P. Tony, M. Wilhelm, *Blood* **2000**, *96*, 384 – 392.
[9] W. K. Born, C. L. Reardon, R. L. O'Brien, *Curr. Opin. Immunol.* **2006**, *18*, 31 – 38.
[10] W. Xu, S. A. Lee, T. G. Kutateladze, D. Sbrissa, A. Shisheva, G. D. Prestwich, *J. Am. Chem. Soc.* **2006**, *128*, 885 – 897.
[11] C. Belmant, E. Espinosa, F. Halary, Y. Tang, M. A. Peyrat, H. Sicard, A. Kozikowski, R. Buelow, R. Poupot, M. Bonneville, J. J. Fournié, *FASEB J.* **2000**, 1669 – 1670.
[12] H. T. Chen, M. F. Neerman, A. R. Parrish, E. E. Simanek, *J. Am. Chem. Soc.* **2004**, *126*, 10044 – 10048.
[13] G. Bansal, J. E. I. Wright, C. Kucharski, H. Uludag, *Angew. Chem.* **2005**, *117*, 3776 – 3780; *Angew. Chem. Int. Ed.* **2005**, *44*, 3710 – 3714.
[14] N. Launay, A.-M. Caminade, R. Lahana, J.-P. Majoral, *Angew. Chem.* **1994**, *106*, 1682 – 1684; *Angew. Chem. Int. Ed. Engl.* **1994**, *33*, 1589 – 1592.
[15] a) T. R. Krishna, M. Parent, M. H. V. Werts, L. Moreaux, S. Gmouh, S. Charpak, A.-M. Caminade, J.-P. Majoral, M. Blanchard-Desce, *Angew. Chem.* **2006**, *118*, 4761 – 4764; *Angew. Chem. Int. Ed.* **2006**, *45*, 4645 – 4648; b) M. Blanzat, C. O. Turrin, A. M. Aubertin, C. Vidal, A.-M. Caminade, J.-P. Majoral, I. Rico-Lattes, A. Lattes, *ChemBioChem* **2005**, *6*, 2207 – 2213.
[16] N. Launay, A.-M. Caminade, J.-P. Majoral, *J. Organomet. Chem.* **1997**, *529*, 51 – 58.
[17] M. K. Boggiano, G. J. A. A. Soler-Illia, L. Rozes, C. Sanchez, C. O. Turrin, A.-M. Caminade, J.-P. Majoral, *Angew. Chem.* **2000**, *112*, 4419 – 4424; *Angew. Chem. Int. Ed.* **2000**, *39*, 4249 – 4254.
[18] M. Poupot, L. Griffe, P. Marchand, A. Maraval, O. Rolland, L. Martinet, F. E. L'Faqihi-Olive, C. O. Turrin, A.-M. Caminade, J. J. Fournié, J.-P. Majoral, R. Poupot, *FASEB J.* **2006**, *20*, 2339 – 2351.
[19] M. A. Cooper, T. A. Fehninger, M. A. Caligiuri, *Trends Immunol.* **2001**, *22*, 633 – 640.
[20] C. A. Biron, K. B. Nguyen, G. C. Pien, L. P. Cousens, T. P. Salazar-Mather, *Annu. Rev. Immunol.* **1999**, *17*, 189 – 220.
[21] P. Garcia-Penarrubia, F. T. Koster, R. O. Kelley, T. D. McDowell, A. D. Bankhurst, *J. Exp. Med.* **1989**, *169*, 99 – 113.
[22] T. Scharton-Kersten, A. Sher, *Curr. Opin. Immunol.* **1997**, *9*, 44 – 51.
[23] a) J. N. Blattman, P. D. Greenberg, *Science* **2004**, *305*, 200 – 205; b) A. Cerwenka, L. L. Lanier, *Nat. Rev. Immunol.* **2001**, *1*, 41 – 49.
[24] H. G. Klingemann, *Cytotherapy* **2005**, *7*, 16 – 22.
[25] A. Moretta, C. Bottino, M. Vitale, D. Pende, C. Cantoni, M. C. Mingari, R. Biassoni, L. Moreta, *Annu. Rev. Immunol.* **2001**, *19*, 197 – 223.

