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Research Article

In vivo and *in vitro* differences between leukocytic uptake of oligodeoxynucleotides

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Abstract. Development and application of therapeutic oligonucleotides rely on proper analysis of binding and uptake. We have used several model oligodeoxynucleotides (ODNs) to analyze binding/uptake by rat and human leukocytes. Here we describe: (1) differences between *in vivo* and *in vitro* uptake of ODNs to rat leukocytes, (2) differences after injection of lipopolysaccharide (LPS), (3) large *in vitro* differences between primary mononuclear cells in PBS, plasma and blood, and (4) differences of ODN uptake between rat and human leukocytes. Our data show that ODN uptake by primary blood cells was different in PBS, plasma and blood. In addition, LPS treatment increased ODN uptake by leukocytes in blood, indicating that pathological conditions may influence ODN uptake. Furthermore, ODN uptake in rat and human blood is also different, suggesting that preclinical ODN uptake data from rat blood cannot easily be extrapolated to the human condition.

Keywords. Oligodeoxynucleotides, leukocytes, cellular uptake, FACS, blood.

Introduction

The last decades witnessed an enormous increase in information about nucleic acids. The elucidation of many disease-related molecular pathways, together with the sequencing of the human genome, make nucleic acids not only targets for disease intervention but also therapeutics to interfere with disease-related processes. Now nucleic acids find a wide range of applications in fields such as biotechnology, molecular biology, diagnosis and therapy. Nucleic acids therapeutics represents a new opportunity for drug discovery. Based on different mechanisms several groups of therapeutic nucleic acids were developed: antisense nucleic acids, including small interfering RNA (siRNA) and ribozymes; immunomodulatory nucleic acids; and aptamers, structured nucleic acids that form binding pockets for specific ligands. Currently, two therapeutic nucleic acids, *i.e.*, the antisense oligonucleotide Fomivirsen and the aptamer Macugen, are already in the market and a variety of other therapeutic nucleic acids are at different stages of clinical trials [1–3].

While conceptually elegant, preclinical and clinical observations raised several concerns of the nucleic acid application, like low stability and cell permeability [4]. Unmodified phosphodiester (PE) oligonucleotides are easily digested by nuclease so phosphorothioate (PS) oligonucleotides are widely used [5]. PS oligonucleotides, in which the non-bridge oxygen in the phosphate linkage is replaced with sulfur, have greatly increased nuclease resistance, protein binding, cellular uptake and so on [6].

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Usually cellular uptake of oligonucleotides is a crucial step for nucleic acids-based therapeutic agents because nucleic acids are polyanions that cannot passively diffuse across cell membranes. Cellular uptake of oligonucleotides has been studied *in vitro* with cell lines, and the mechanisms for cellular uptake of oligonucleotides are considered as fluid-phase endocytosis at relevant high concentrations (greater than 1 μM), and receptor-mediated endocytosis at lower oligonucleotide concentrations [7, 8]. However, investigations concerning oligonucleotide uptake into primary cells, particularly in biological fluid, like blood, are limited. Following administration of oligonucleotides into the blood, which is a complex mixture of cells, proteins and other elements, oligonucleotides are associated with plasma proteins, like albumin, which may result in the entrapment of oligonucleotides in large particles and make the cellular uptake of oligonucleotides in blood different from that *in vitro* [9].

Circulating leukocytes are the major element of blood exposed directly to systemically administered oligonucleotides. Leukocytes are also essential for eliciting immune responses and served as the major targets of immunomodulatory oligonucleotides [10–12]. Furthermore, leukocytes form target cells in clinical studies testing antisense oligonucleotides as therapeutics for inflammatory disease, such as rheumatoid arthritis and Crohn's disease [13], for hematological malignancies [14] and for human immunodeficiency virus infection [15]. Therefore, we investigated oligodeoxynucleotide (ODN) cellular uptake by primary leukocytes in blood that may resemble more closely the *in vivo* condition.

The object of the present study was to study the influence of plasma on cellular ODN uptake by primary mononuclear cells and ODN uptake by rat and human primary leukocytes in blood. Furthermore, ODN uptake in blood from LPS- or PBS-treated rats was compared to examine whether pathological conditions can alter ODN cellular uptake in blood. Four 15-bp homopolymers ODNs and PE and PS immunostimulatory ODNs that have hexameric motifs consisting of central unmethylated CpG dinucleotides were used in this study.

Materials and methods

ODNs. ODNs used in this study were synthesized by MWG Biotech (Ebersberg, Germany). Sequences of ODNs are as follows: poly(dA): 5′-AAAAAAAAAAAAAAA-3′, poly(dT): 5′-TTTTTTTTTTTTTTT-3′, poly(dC): 5′-CCCCCCCCCCCCCCC-3′, poly(dG): 5′-GGGGGGGGGGGGGGG-3′, and CpG: 5′-TCCATGACGTTCCTGACGTT-3′. A PS backbone CpG ODN was also used and termed PS-CpG. Correspondingly, the unmodified PE backbone CpG ODN was named PE-CpG. All ODNs were labeled with FITC at the 5′ end.

Antibodies. For rat leukocyte differentiation antigen detection, RPE-conjugated mouse anti-rat monoclonal antibodies were used to detect T cells (CD3, BD Pharmingen, Heidelberg, Germany), B cells (OX33, Serotec, Oxford, UK) or monocytes (ED9, Serotec, Oxford, UK). To identify human leukocyte subpopulations, following mouse anti-human monoclonal antibodies were applied to detect T cells (CD3, RPE-conjugated, Serotec), B cells (CD22, RPE-conjugated, Serotec), monocytes (CD14, RPE-conjugated, Serotec) or granulocytes (CD16, RPE-CY5-conjugated, Serotec). RPE-conjugated mouse IgG isotype control was from BD Pharmingen.

Rats. Male Lewis rats (8–10 weeks, 170–200 g, Charles River, Sulzfeld, Germany) were housed under a 12-h light–12-h dark cycle and with free access to food and water. All animal procedures were in accordance with a protocol approved by the local Administration District Official Committee. All efforts were made to minimize the number of animals and their suffering. To study LPS-induced changes of ODN uptake, rats received intraperitoneal injections of LPS from *Escherichia coli* serotype 026:B6 (Sigma-Aldrich Chemie, Munich, Germany; 5 mg/kg body weight in 1 ml PBS) or PBS (control group) and were killed 24 h later.

Cytofluorometric analysis of ODN uptake by primary rat mononuclear cells. Rat EDTA-blood was drawn by cardiac puncture under anesthesia and mononuclear cells from whole blood were separated using BD Vacutainer™ CPT™ according to the manufacturer's instructions (BD, Franklin Lakes, USA). Trypan blue staining was used to detect cell viability and only samples with more than 99 % living cells were used. After counting, 1 μl FITC-labeled ODNs (100 μM) was added to 100 000 cells/100 μl PBS or rat plasma, which was derived from the same donor of mononuclear cells, and incubated for 30 min at room temperature in the dark. Subsequently, cells were fixed with ERYTHROLYSE (Serotec), which contains paraformaldehyde, according to the manufacturer's instructions and then washed twice with washing buffer (PBS pH 7.1 containing 10 mg/ml BSA, 1 mg/ml sodium azide and 50 μg/ml yeast tRNA). After washing, cells were suspended in 250 μl FACS buffer (PBS pH 7.1 containing 10 mg/

ml BSA and 1 mg/ml sodium azide) and analyzed by FACS. Mononuclear cells were gated by forward and sideward scatter.

For investigation of ODN uptake, it is important to distinguish internalized ODNs from cell-surface bound ODNs. DNase treatment of cells and washing the cells in high salt/low pH buffers (such as glycine, pH 2.0) to remove cellular adhered material have been applied but the efficacy of either of these methods is open to question and both may be toxic to the cells [16]. A recommended and more physiological approach is to add a competitor, which is not toxic to cells and will remove virtually all cell surface-bound PS ODNs [16]. In our study, a high concentration tRNA was used as unspecific competitor, and the remaining fluorescence signals were regarded as from inside of the cells.

Cytofluorometric analysis of ODN uptake by leukocytes in blood *in vitro*. Rat EDTA-blood was drawn by cardiac puncture under anesthesia (n=3) and human peripheral EDTA-blood samples were obtained from healthy donors (n=5). An aliquot of 100 µl whole blood was incubated at room temperature for 30 min with 1 µl FITC-labeled ODNs (100 µM). Thereafter, erythrocytes were lysed with ERYTHROLYSE (Serotec) according to the manufacturer's instructions. Cells were washed twice with washing buffer, suspended in 250 µl FACS buffer and analyzed by a FACScan (Becton Dickinson, Überlingen, Germany). Leukocytes were gated by forward and sideward scatter.

To assess ODN uptake by different cell subpopulations, monoclonal antibodies were added to 100 µl whole blood and incubated at room temperature for 30 min. Thereafter, 1 µl FITC-labeled ODN (100 µM) was added and incubated for another 30 min, and the steps were as described above were followed.

Fluorescence microscopy. To analyze of intracellular ODN distribution, leukocytes treated as described above for FACS analysis, were centrifuged (1000 rpm, 5 min) at room temperature, transferred onto slides, mounted in a mounting medium containing DAPI (Vector Laboratories, Inc., Burlingame, USA) and then observed by fluorescence microscopy. All experiments were performed in triplicate.

ODN injection and flow cytometric analysis of uptake. FITC-labeled poly(dT) ODNs (0.5 µmol) were dissolved in 1 ml PBS and injected intravenously. After 10 min, rats were killed and blood was taken by heart puncture (n=3). Whole blood (100 µl) was incubated at room temperature for 30 min with antibodies. Thereafter, erythrocytes were lysed with ERYTHROLYSE (Serotec) according to the manufacturer's instructions. Cells were washed twice with washing buffer, suspended in 300 µl FACS buffer and analyzed by FACS.

Data evaluation and statistics. Fluorescence positive cells were defined as cells presenting higher fluorescence than background, defined as fluorescence of cells incubated with unconjugated ODN or isotype control antibody. An unpaired t-test was performed to compare changes between treated and untreated groups (GraphPad Prism 4.0 for windows). For all statistical analyses, significance levels were set at $p < 0.05$.

Results

***In vivo* ODN uptake by rat leukocytes.** Here we have used simple model ODNs to study *in vivo* ODN uptake in blood of normal rats and after LPS injection. FITC-labeled poly(dT) ODNs were intravenously injected 1 day after intraperitoneal injection of LPS (5 mg/kg in 1 ml PBS) or PBS. After 10 min, blood was taken (with EDTA as an anti-coagulant) and stained with RPE-conjugated monoclonal antibodies. Thereafter, red blood cells were lysed, leukocytes were fixed, cell surface-bound ODNs were removed by unspecific binding competition using washing buffer with a high tRNA concentration, and cellular uptake analyzed by flow cytometry. The results of one representative experiment of three experiments are shown in Figure 1.

In PBS-treated rats, FITC-labeled poly(dT) ODNs were internalized by leukocytes, particularly by monocytes (Fig. 1). Following LPS treatment, *in vivo* poly(dT) ODN uptake by leukocytes was greatly increased compared to PBS control (Fig. 1). LPS treatment also relatively decreased circulating T ($CD3^+$) and B ($OX33^+$) cells, but greatly increased circulating monocytes ($ED9^+$) (Fig. 1).

***In vitro* ODN uptake by rat leukocytes.** ODN uptake in normal rat blood by primary leukocytes was also analyzed *in vitro*. FITC-labeled poly(dA), poly(dT), poly(dC), poly(dG), PE-CpG and PS-CpG ODNs were used. Fresh rat blood were stained by different monoclonal antibodies and then incubated with FITC-labeled ODNs (final concentration 1 µM) for 30 min at room temperature. Subsequently, erythrocytes were lysed, leukocyte were fixed, cell surface-bound ODNs were removed by washing buffer, and the uptake of ODNs by leukocytes analyzed by flow cytometry. The results of one representative experiment of three experiments are shown in Figure 2.

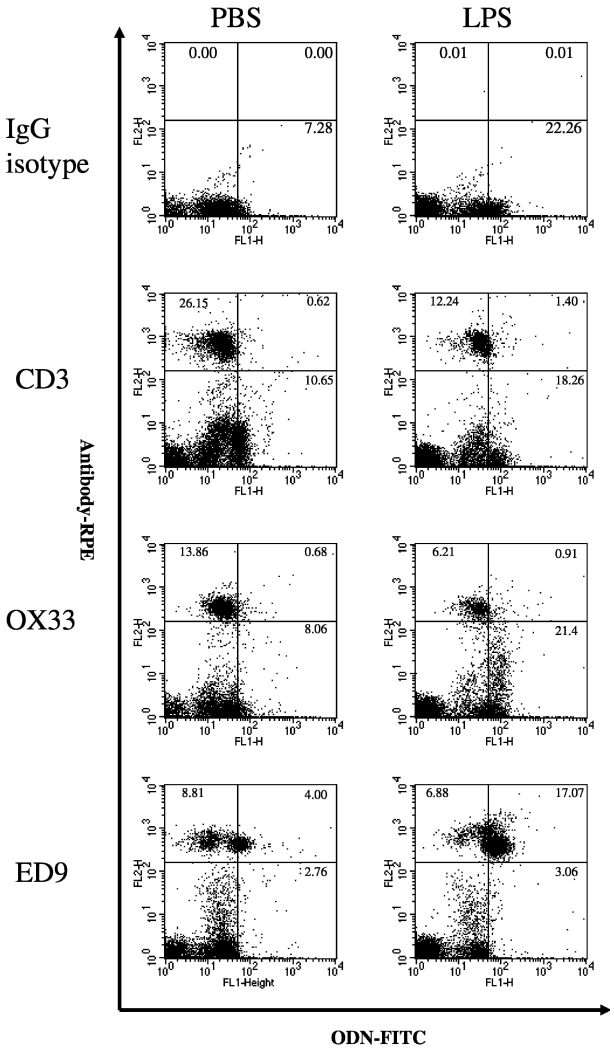

Figure 1. *In vivo* uptake of oligodeoxynucleotides (ODNs) by leukocytes. At 1 day after intraperitoneal injection of PBS (1 ml, control group) or of LPS (5 mg/kg in 1 ml PBS), FITC-labeled poly(dT) ODNs were injected intravenously. After 10 min, blood (with EDTA as an anti-coagulant) was taken and incubated with different RPE-conjugated antibodies for 30 min at room temperature. Thereafter, ERYTHROLYSE, which contains paraformaldehyde, was added to lyse red blood cells and to fix leukocytes. After washing with washing buffer containing high concentration of yeast tRNA, cellular uptake of ODNs was analyzed by flow cytometry. In PBS-injected rats, FITC-labeled poly(dT) ODNs were internalized by leukocytes, particularly by monocytes. Following LPS treatment, poly(dT) ODN uptake by leukocytes was greatly increased compared to PBS control. LPS treatment also relatively decreased circulating T ($CD3^+$) and B cells ($OX33^+$), but greatly increased circulating monocytes ($ED9^+$). Numbers in each quadrant represent percentage of cells in the quadrant.

Although T ($CD3^+$) and B cells ($OX33^+$) represent major populations of leukocytes in rat blood, they barely internalized ODNs. For all our examined ODNs, 0.7–4 % T cells and 0.6–6.7 % B cells were $FITC^+$. Monocytes ($ED9^+$ cells) were the major cell subpopulation for uptake of poly(dA), poly(dT), poly(dC), PE-CpG and PS-CpG ODNs. Poly(dA), poly(dT) and PE-CpG ODNs had the highest uptake efficiency and about 91 %, 73 % and 63 % $FITC^+$ cells were monocytes, respectively. Therefore, monocytes were the major leukocyte subpopulation for ODN uptake by rat leukocytes in blood *in vitro*, identical to the *in vivo* observation described above.

Cellular distribution of FITC-labeled ODNs (nuclei were counterstained with DAPI) was analyzed by fluorescence microscopy. Randomly selected $FITC^+$ cells are shown in Figure 3. ODNs mainly localized to the cytoplasm and showed a spotted distribution, similar to our previous observations in cultured cells [17]. According to the morphology of the nucleus, lymphocytes (Fig. 3D), monocytes (Fig. 3B) and granular cells (Fig. 3A, C, E and F) were all identified as taking up FITC-ODNs.

In addition, whether FITC-labeled ODNs could be trapped in red blood cells was analyzed. After adding FITC-labeled ODN *in vitro* to whole rat blood, incubation and washing, samples were observed by fluorescence microscopy without erythrocyte lysing. As shown in Figure 3G, FITC signals were seen not only in nucleated cells but also in erythrocytes.

LPS injection increased ODN uptake *in vitro* by leukocytes. As therapeutically used ODNs would be applied under pathological conditions, we further analyzed ODN uptake in whole blood of LPS-treated rats. Rats were injected intraperitoneally with LPS (5 mg/kg in 1 ml PBS, $n=3$) or PBS (1 ml, $n=3$) and blood was taken for analysis of ODN uptake by flow cytometry 24 h later. Except for PS-CpG ODNs ($p>0.05$), all other tested FITC-labeled ODNs [poly(dA), poly(dT), poly(dC), poly(dG) and PE-CpG] ($p<0.05$) exhibited significantly increased cellular uptake in whole blood of LPS-treated rats compared to PBS-treated control rats (Fig. 4A). Changes of leukocyte subpopulations were also analyzed following LPS-treatment in rats. As shown in Figure 4B, LPS treatment relatively decreased circulating T cells ($CD3^+$, $p<0.05$) and B cells ($OX33^+$, $p<0.05$) but greatly increased circulating monocytes ($ED9^+$, $p<0.05$).

Furthermore, cellular ODN uptake by different leukocyte subpopulations in blood of LPS- or PBS-treated rats was investigated. The results of one representative experiment of three experiments are shown in Figure 5. While LPS treatment significantly decreased percentage of T and B cells in rat blood, following LPS treatment, T cells ($CD3^+$) and B cells ($OX33^+$) took poly(dT) ODNs up with higher efficiency than PBS control (Fig. 5). For monocytes ($ED9^+$), LPS treatment greatly increased their percentage in blood and their uptake of poly(dA), poly(dT), poly(dC), poly(dG) and PE-CpG ODNs were significantly enhanced.

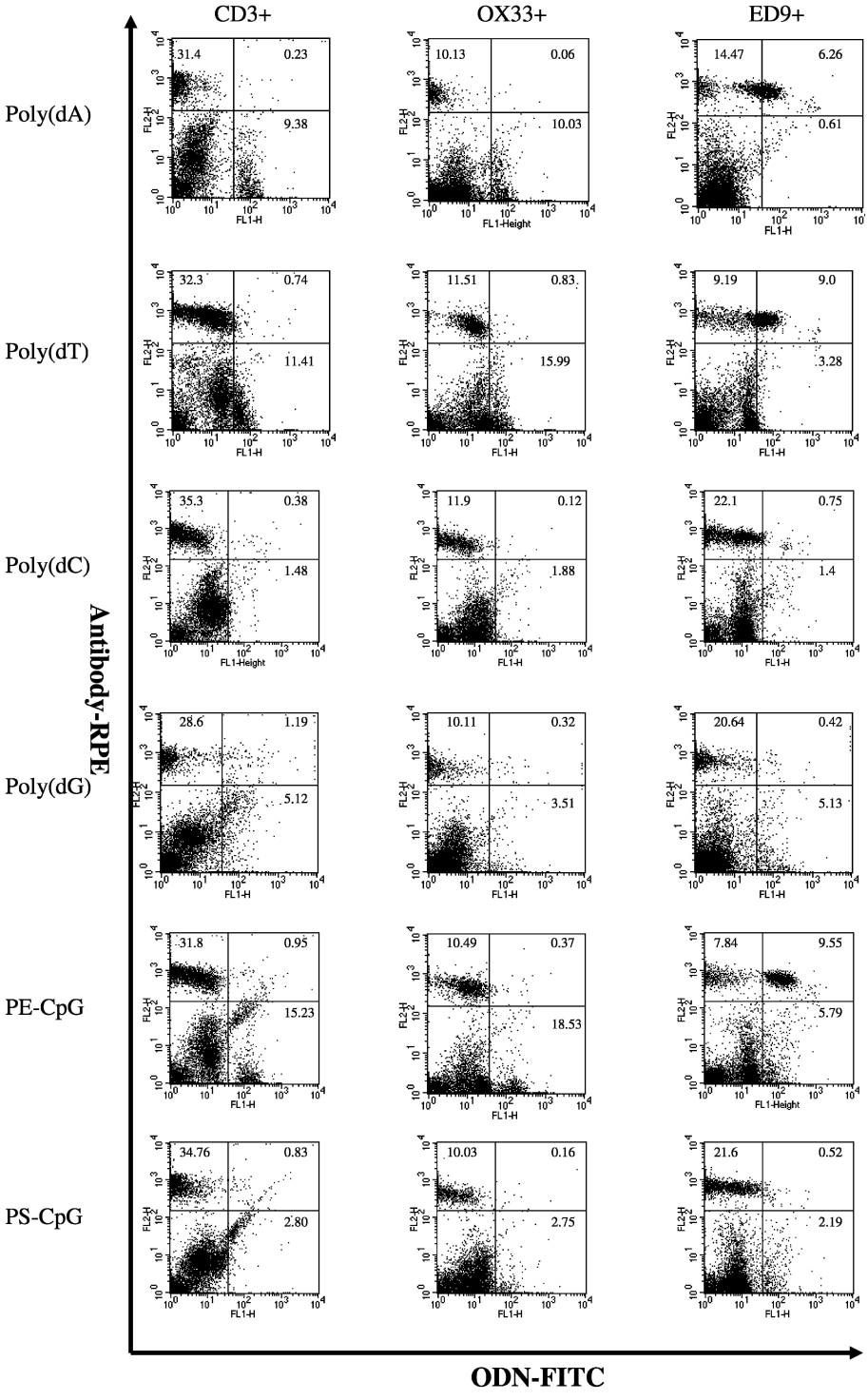

Figure 2. *In vitro* ODN uptake in rat blood by leukocytes. EDTA anti-coagulated fresh rat blood was used. Monoclonal antibodies (CD3 for T cells, OX33 for B cells and ED9 for monocytes) were added to 100 µl rat blood and incubated for 30 min at room temperature. Thereafter, 1 µl FITC-labeled ODN (100 µM) was added and incubated for another 30 min. Subsequent procedures were as described in Figure 1. The results of one representative experiment of three experiments are shown. Monocytes were identified to be the major leukocyte subpopulation for ODN uptake in rat blood *in vitro*. Numbers in each quadrant represent percentage of cells in the quadrant.

ODN uptake by primary rat mononuclear cells in PBS, plasma and whole blood. FITC-labeled ODNs, poly(dA), poly(dT), poly(dC), poly(dG), PE-CpG and PS-CpG, were incubated for 30 min with PBS or plasma diluted primary rat mononuclear cells (100 000 cells/100 µl) or rat whole blood (100 µl) to a final concentration of 1 µM/µl. Thereafter, ERYTHROLYSE, which contains paraformaldehyde, was added to lyses red blood cells and fix leukocytes. After washing, cellular uptake of ODNs was analyzed by flow cytometry. Results of one representative experiment of three experiments are shown in Figure 6. For each experiment, three parallel samples were prepared for every ODN.

PBS-diluted primary rat mononuclear cells had the highest cellular uptake of poly(dC) and PS-CpG

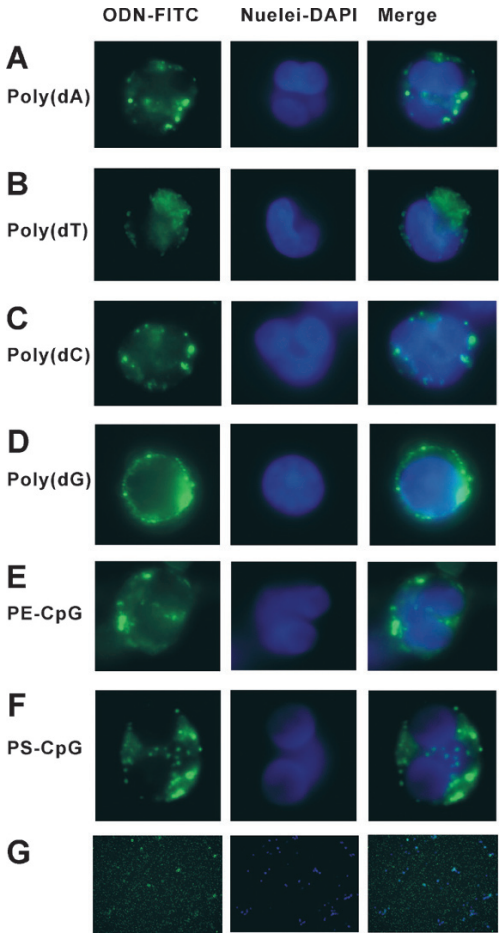

Figure 3. Granular pattern of ODN localization, predominantly to the cytoplasm, of leukocytes. Following analysis of *in vitro* ODN uptake in whole rat blood by flow cytometry, cells were collected, transferred onto slides, mounted in a mounting medium containing DAPI and then observed by fluorescence microscopy. FITC$^+$ cells were seen for each of the ODNs. All FITC-labeled ODNs were localized mainly in the cytoplasm and showed a spotted distribution pattern 30 min after incubation. According to the morphology of the nucleus, lymphocytes (*D*), monocytes (*B*) and granular cells (*A*, *C*, *E* and *F*) were all identified to take-up ODNs. (*G*) To analyze whether FITC-labeled ODNs could be trapped by red blood cells, samples were observed by fluorescence microscopy without erythrocyte lysing following addition of FITC-labeled ODN to rat blood, incubation and washing. FITC signals were seen not only in nucleated cells but also in erythrocytes. Original magnification: A–F ×1000, G ×200.

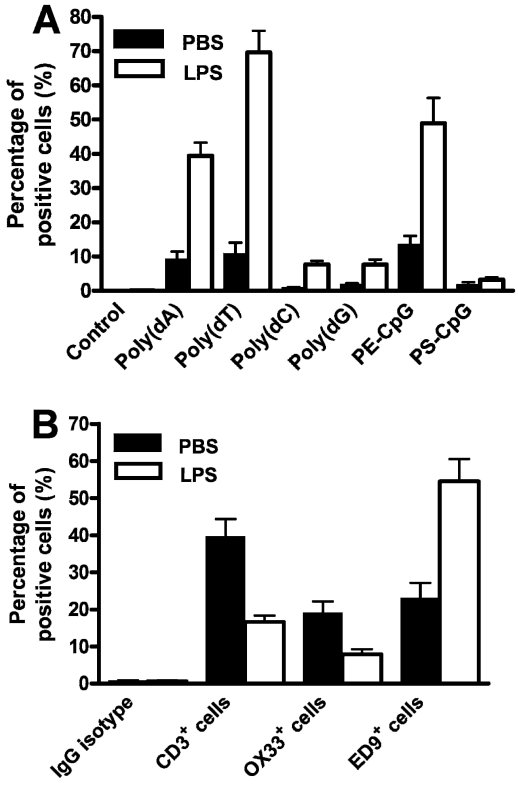

Figure 4. LPS administration induces *in vivo* increase in ODN uptake by rat leukocytes. Rats were intraperitoneally injected with LPS (5 mg/kg in 1 ml PBS, $n=3$) or PBS (1 ml, $n=3$) and blood was taken for analysis of ODN uptake by flow cytometry 24 h later as described in Figure 2. Except for PS-CpG ODNs ($p>0.05$), other tested FITC-labeled ODNs [poly(dA), poly(dT), poly(dC), poly(dG) and PE-CpG] ($p<0.05$) labeled a significantly increased numbers of leukocytes in blood of LPS-treated rats compared to PBS-treated rats (*A*). LPS treatment slightly decreased circulating T cells (CD3$^+$) and B cells (OX33$^+$), but greatly increased circulating monocytes (ED9$^+$) (*B*).

ODNs. Although the sequence was the same, the unmodified CpG ODNs (PE-CpG) were much less efficiently internalized by mononuclear cells compared to PS-CpG ODNs. Poly(dA) and PE-CpG ODNs had the lowest uptake and poly(dT) and poly(dG) ODNs had the comparable level of uptake to poly(dC) by primary rat mononuclear cells (Fig. 6). For plasma-diluted primary rat mononuclear cells, while their cellular uptake profile of all used ODNs was the same as in PBS-diluted primary mononuclear cells, their cellular uptake efficiency of all used ODNs was generally reduced compared to PBS diluted primary mononuclear cells, indicating that plasma could reduce ODN cellular uptake (Fig. 6).

In general, cellular uptake of ODNs was greatly reduced in blood compared to in PBS or plasma, except for poly(dA) and PE-CpG ODNs (Fig. 6). Interestingly, the uptake profile of ODNs that we used in whole rat blood differed from that in primary mononuclear cells in PBS or plasma (Fig. 6). In whole rat blood, poly(dA) and PE-CpG ODNs exhibited much higher uptake efficiency than poly(dC), poly(dG) and PS-CpG ODNs, which was opposite for primary mononuclear cells in PBS and plasma.

ODN uptake in human whole blood *in vitro*. Human blood was drawn from healthy donors *via* basilic vein. Uptake efficiency of different FITC-labeled ODNs was tested in human blood *in vitro* using the same methods for rat blood. Uptake profile of ODNs that

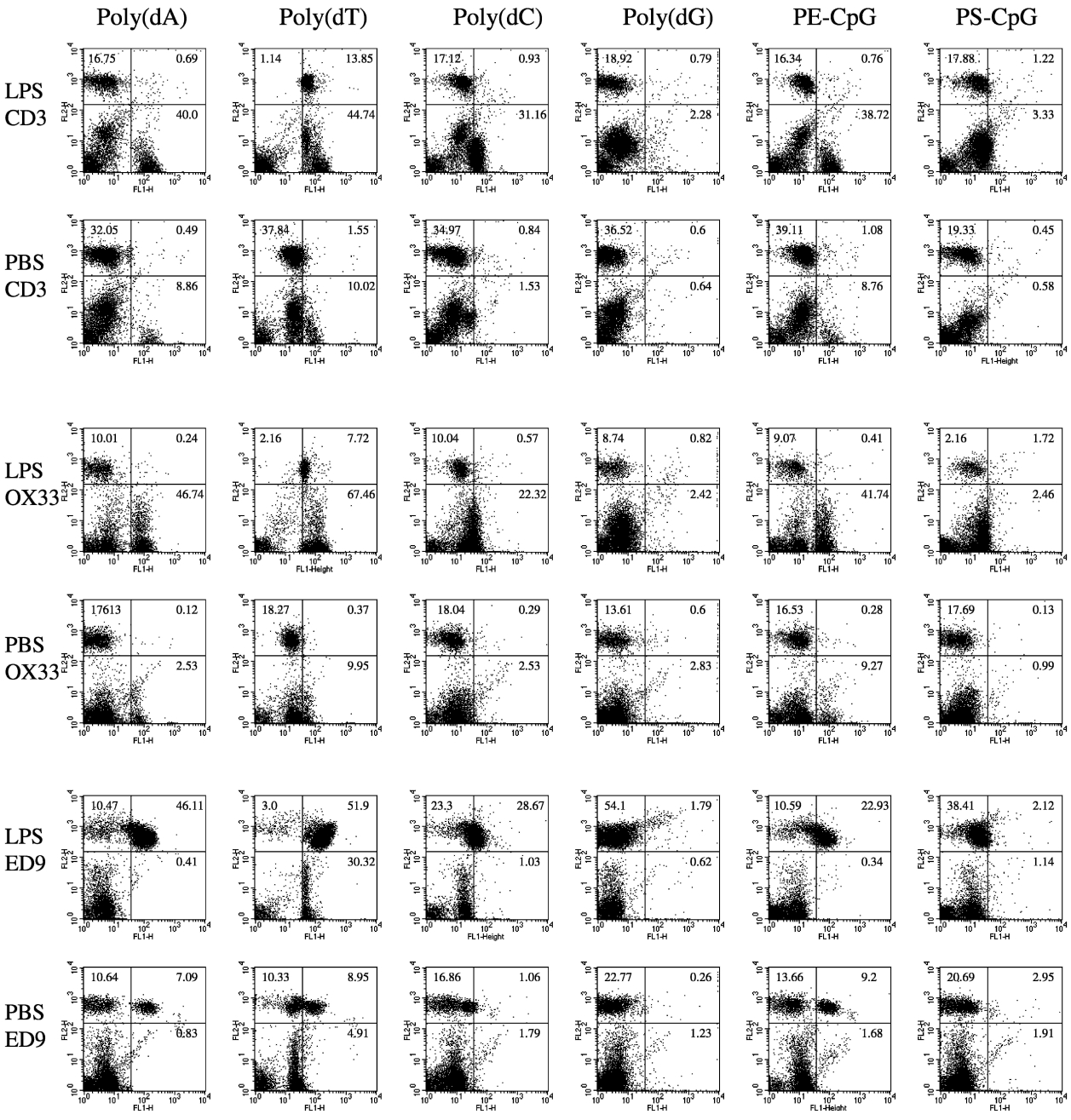

Figure 5. Comparison of ODN uptake by different leukocyte subpopulations in blood of LPS- or PBS-treated rats. Although LPS treatment significantly decreased percentage of T and B cells in rat blood, after LPS treatment T cells ($CD3^+$) and B cells ($OX33^+$) exhibited higher uptake efficiency of poly(dT) ODNs. For monocytes ($ED9^+$), LPS treatment greatly increased their percentage in blood and their uptake of poly(dA), poly(dT), poly(dC), poly(dG) and PE-CpG ODNs compared to PBS treatment. Numbers in each quadrant represent percentage of cells in the quadrant. Results of one representative experiment of three experiments are shown.

we used in human blood was different from that in rat blood (Fig. 7). Generally, besides poly(dG) ODNs, other applied ODNs were more efficiently internalized by leukocytes in human than in rat blood. In addition, poly(dC) and PS-CpG ODNs, which had the lowest uptake efficiency in rat blood, showed comparable uptake efficiency to poly(dA), poly(dT) and PE-CpG ODNs in human blood. However, as for rat blood, poly(dG) ODNs were barely taken up by leukocytes in human blood.

The major cell subpopulations for FITC-labeled ODN uptake in human blood were further identified using monoclonal antibodies conjugated with RPE or RPE-Cy3. The results of one representative experiment of four are shown in Figure 8. The major human leukocyte subpopulation was granulocytes ($CD16^+$ cells,

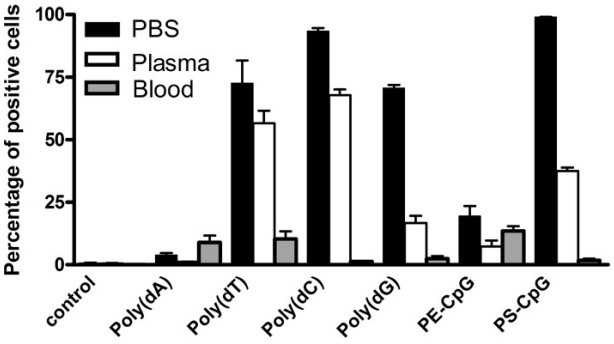

Figure 6. Comparison of *in vitro* ODN uptake by primary rat mononuclear cells. FITC-labeled ODNs, poly(dA), poly(dT), poly(dC), poly(dG), PE-CpG and PS-CpG, were incubated for 30 min with PBS or plasma diluted primary rat mononuclear cells (100 000 cells/100 µl) or rat whole blood (100 µl) to a final concentration of 1 µM/µl. Thereafter, ERYTHROLYSE, was added. After washing with washing buffer containing high concentration of yeast tRNA, cellular uptake of ODNs was analyzed by flow cytometry. Results of one representative experiment of three experiments are shown. For each experiment, three parallel samples were prepared for every ODN. ODN uptake efficiency by primary mononuclear cells in plasma was reduced compared to PBS. Furthermore, ODN uptake profile by leukocytes in blood differed from in PBS and plasma, and poly(dT), poly(dC), poly(dG) and PS-CpG ODN uptake efficiency was much lower in blood compared to PBS or plasma.

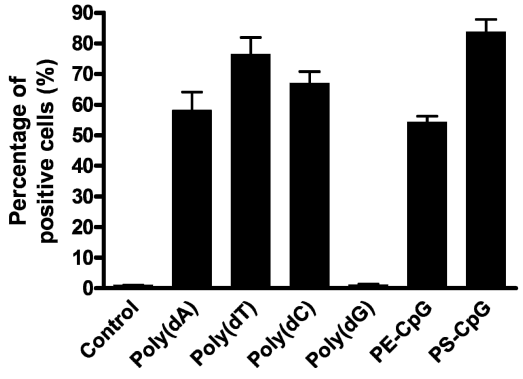

Figure 7. *In vitro* uptake of ODNs in human whole blood. Human blood was drawn from healthy donors. ODN uptake was tested in human blood *in vitro* using the same method as for rat blood. Generally, besides poly(dG), all other ODNs were more efficiently internalized by leukocytes of human than rat blood. Further, the uptake profile of ODNs in human blood was different from rat blood.

more than 50 %), which is in accordance with previous reports. As shown in Figure 8, T cells ($CD3^+$), B cells ($CD22^+$), monocytes ($CD14^+$) and granulocytes ($CD16^+$) rarely took up poly(dG) ODNs. For other examined ODNs [poly(dA), poly(dT), poly(dC), PE-CpG and PS-CpG ODNs], more than 70 % $FITC^+$ cells were identified to be granulocytes ($CD16^+$). Also, more than 90 % granulocytes were $FITC^+$ cells for poly(dA), poly(dT), poly(dC), PE-CpG and PS-CpG ODNs. These observations identified granulocytes to be the major leukocyte subpopulation for cellular uptake in human blood. Monocytes, which were the major cells for ODN uptake in rat blood, contributed to poly(dT) and PS-CpG ODN cellular uptake (Fig. 8). Both T and B cells internalized PS-CpG ODNs. However, only T and not B cells internalized poly(dT) ODNs.

Discussion

Cellular uptake of ODN is a crucial aspect for most ODN-based therapeutic agents and a limiting step of ODN activity. Here we have analyzed cellular uptake of a panel of ODNs by leukocytes. *In vitro* and *in vivo*, as well as species-specific, sequence-specific, or modification-specific differences in uptake were observed, which were further influenced by pathological states. *In vitro* uptake of ODNs by primary rat mononuclear cells was reduced in plasma compared to PBS. Thus, binding/uptake studies without the presence of plasma proteins yielded different results. In rat blood, ODN uptake by leukocytes was greatly reduced compared to PBS or plasma, and the major cells for ODN internalization were monocytes ($ED9^+$). *In vitro* and *in vivo*, ODN uptake by leukocytes in rat blood was significantly increased 24 h following intraperitoneal injection of LPS. In human blood, the uptake profile of ODNs differed from that in rat blood and the major cells for ODN uptake were granulocytes.

ODNs bind to plasma proteins, such as albumin, α-1-glycoprotein, and others [6, 9, 18]; however, it remains to be determined whether such binding can influence ODN cellular uptake. In our study, ODN uptake by primary rat mononuclear cells in plasma was lower than in PBS, particularly for poly(dG) and PS-CpG ODNs. ODNs are polyanions. This makes it difficult for ODNs to simply diffuse through cell membranes. While the mechanisms of ODN uptake are still far from clear, receptor-mediated endocytosis is supported by some studies [7, 8]. Therefore, high-binding affinity of ODN to proteins may increase ODN binding to cells, resulting into enhanced cellular uptake. However, in blood, such binding may also limit ODN uptake by reducing effective ODN concentration. It should, however, been noted that cultured cell lines may have a different ODN uptake profile, leading to contrasting observations. Wasan et al. [19] and Bai et al. [20] showed that plasma could favorably affect ODN cellular uptake using cultured melanoma and leukemia cell lines, respectively.

Interestingly, ODN uptake in rat blood differed from that in plasma or PBS. In rat blood, ODN uptake efficiency decreased and the ODN uptake profile of different ODNs was altered compared to PBS and plasma. Except for poly(dA) and PE-CpG ODNs,

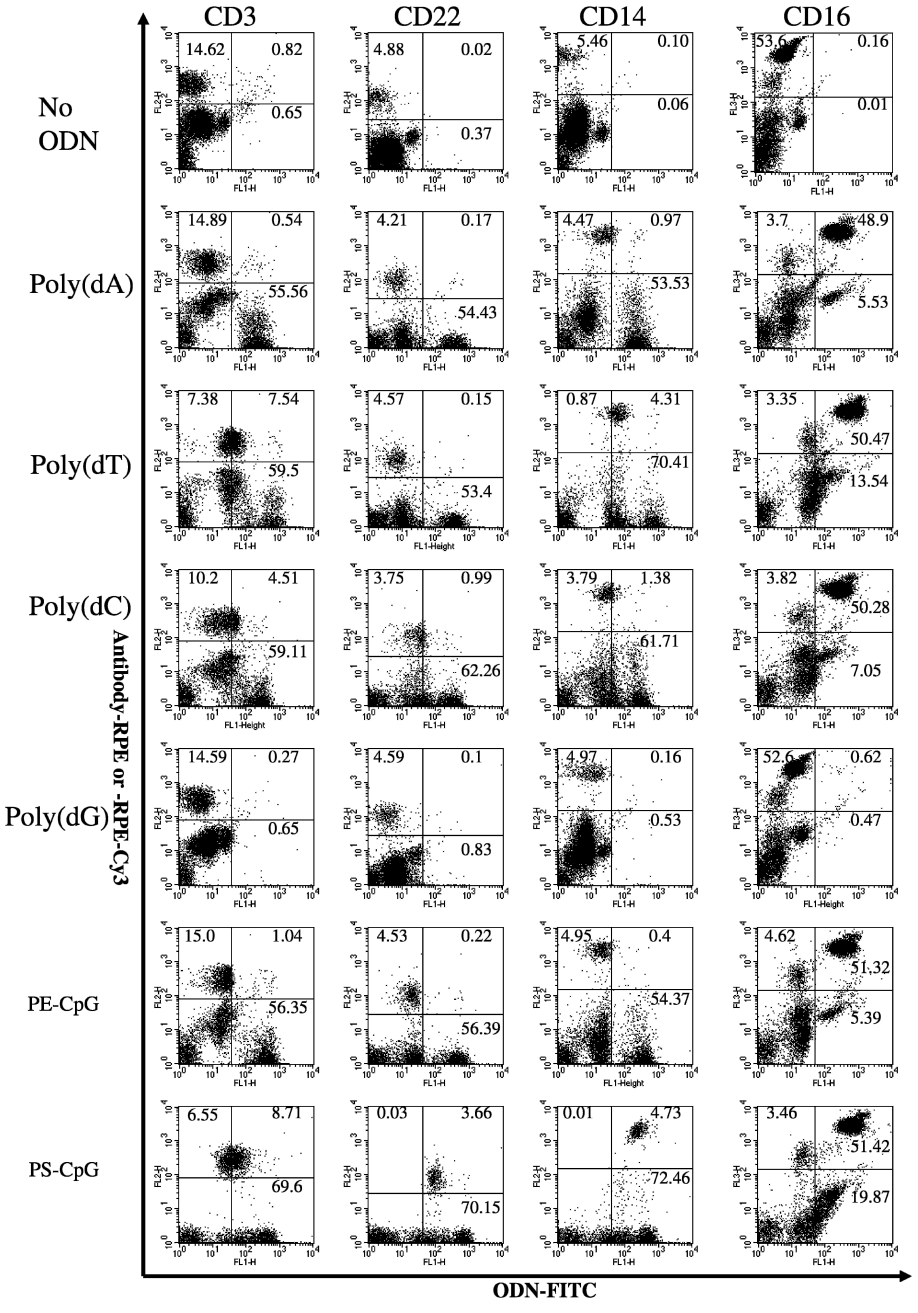

Figure 8. Contributions of different cell subpopulations to ODN uptake in human blood. Monoclonal antibodies (CD3 for T cells, CD22 for B cells, CD14 for monocytes and CD16 for granulocytes) were applied; the method was the same as that described for rat blood in Figure 2. Granulocytes were identified as the major human leukocyte subpopulation taking up ODNs. Numbers in each quadrant represent percentage of cells in the quadrant.

which showed the lowest uptake efficiency in PBS and plasma, uptake efficiency of the other four ODNs, which had high uptake efficiency in PBS and plasma, was greatly reduced in blood compared to PBS and plasma. Compared to plasma, erythrocytes are unique in blood. Erythrocytes are potential biocompatible vectors for different bioactive substances, including oligonucleotides [21, 22]. Red blood cells have been successfully used to deliver modified oligonucleotides [23]. In our study, following incubation of FITC-labeled ODNs with rat blood and subsequent washing, strong FITC signals were observed in erythrocytes (Fig. 3), indicating that part of ODNs were trapped in red blood cells in rat blood, which might decrease the effective concentration of ODNs in blood, resulting in reduced ODN cellular uptake.

The therapeutic efficiency and specificity of experiments using therapeutic oligonucleotides *in vivo* in animals or humans would be improved if they were preferentially taken up by the cells of interest. Therefore, it is important to know whether leukocytic ODN uptake is homogenous or heterogeneous. We observed that ODN uptake by leukocytes of rat and human peripheral blood was heterogeneous for different cell types, which confirmed previous observations [24–28]. In peripheral blood, ODN uptake was gen-

erally low in fresh lymphocytes (T and B cells) of rats and humans. This was in line with reports of Ivesen et al. [24] and Zhao et al. [26], respectively. In rat peripheral blood, monocytes contributed to most of the ODN uptake [25]; however, in human peripheral blood, granulocytes were responsible for major ODN uptake. For cultured human mononuclear cells, monocytes were identified as the major cell type with ODN uptake [26–28], but the major primary leukocytes for ODN uptake in human blood have not been investigated yet. Granulocytes constitute the majority of human leukocytes in blood stream (50–70 % in adult). Similar to monocytes, one of the predominant roles of granulocytes is phagocytosis of pathogens. The great variability of ODN uptake of different cell types may be related to an endocytic activity or a unique distribution of cell surface ODN ligands [7, 8]. It is well know that monocytes and granulocytes have a greater endocytosis activity than lymphocytes [29].

While cellular ODN uptake is not considered to be sequence dependent, our data showed that ODN sequence could influence ODN uptake efficiency. Six different simple model ODNs were applied in this study. Although the length was the same, uptake efficiency of poly(dA) ODN was much lower than that of poly(dT), poly(dC) and poly(dG) in PBS and plasma. ODN modification is an important factor to affect ODN uptake efficiency. PS modification is known to increase nuclease resistance, protein-binding affinity and *in vitro* cellular uptake [6, 9]. *In vitro*, PS ODNs showed much higher uptake efficiency than their PE counterparts [7, 8, 12, 17, 30], which is in accordance with our observations here.

In addition, we found that ODN uptake by rat leukocytes was inducible by LPS. Intraperitoneal injection of LPS greatly increased ODN uptake in blood *in vitro* and *in vivo*. As monocytes in the circulation are greatly induced by LPS, and this in part explains the increased ODN uptake. Furthermore, activation of mononuclear cells by LPS may also contribute to enhanced ODN uptake. Incubation of cultured mononuclear cells with LPS has been shown to increase ODN uptake [24, 25, 31]. In addition, activation of bone marrow by growth factors also significantly enhanced ODN uptake [26]. It is known that LPS induces activation and proliferation of lymphocytes and monocytes to synthesize and release inflammatory cytokines that may progress to septic shock *in vivo* [32–35], also indicating that LPS may activate mononuclear cells to increase ODN uptake. LPS is a component of the outer membranes of Gram-negative bacteria and has been long used to mimic bacterial infection [36]. Thus, it is clear that leukocytic ODN uptake is present in normal rats, but varies under pathological conditions. This fact should be kept in mind when designing preclinical studies.

ODN uptake in blood in rats *in vivo* has also been investigated. FITC-labeled poly(dT) ODNs were injected intravenously and ODN uptake by leukocytes was analyzed. The results from this *in vivo* study were similar to those of the *in vitro* study in that (1) monocytes were the major cell type for ODN uptake, (2) LPS greatly increase ODN uptake, and (3) lymphocytes barely internalized ODNs. These data not only confirm the *in vitro* data but also indicate that *in vitro* incubation of ODN with whole blood would be an option for investigation of ODN uptake.

ODN uptake in human blood was also investigated. Interestingly, uptake differed from that in rat blood in that: (1) most ODN had higher uptake efficiency in human blood than in rat blood, (2) uptake profile of ODN was different, and (3) the major cell type for ODN uptake in human blood was granulocytes and not monocytes. Large differences among cell types with respect to ODN uptake have been reported, not only for cell lines [18, 37] but also for primary cells [38].

Thus, it appears that *in vitro* uptake studies with ODNs yield different results, with incubation of ODNs in whole blood samples being closest to the *in vivo* situation. Sequence- and modification-dependent differences make it necessary to evaluate each type of ODN separately. The differences between rat and human binding patterns limit simple extrapolation of rat data to human. These findings are helpful for rational design, evaluation and application of ODN-based therapeutic strategies.

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