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Toll like receptor mediated immune stimulation can be visualized in vivo by [^{18}F]FDG-PET

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ABSTRACT

Introduction: High uptake of [^{18}F]-2-fluorodeoxyglucose ([^{18}F]FDG) by inflammatory cells is a frequent cause of false positive results in [^{18}F]FDG-positron-emission tomography (PET) for cancer diagnostics. Similar to cancer cells, immune cells undergo significant increases in glucose utilization following activation, e.g., in infectious diseases or after vaccination during cancer therapy. The aim of this study was to quantify certain immune effects in vitro and in vivo by [^{18}F]FDG-PET after stimulation with TLR ligands and specific antibodies.
Methods: In vivo [^{18}F]FDG-PET/magnetic resonance imaging (MRI) and biodistribution was performed with C57BL/6 mice immunized with CpG or LPS. Cellular [^{18}F]FDG-uptake assays were performed with B cells and T cells or with whole spleen cells after stimulation with CpG, LPS and anti-CD3/CD28. In vitro and in vivo activation of B and T cells was examined by concomitant FACS analysis to correlate immune cell activation with the strength of [^{18}F]FDG accumulation.
Results: We could show that TLR mediated activation of B cells increases [^{18}F]FDG uptake, and that B cells show faster kinetics and greater effect than T cells stimulated by the CD3/CD28 pathway. In the whole spleen cell population the [^{18}F]FDG signal was triggered mainly by the activation of B cells, corresponding closely to expression of typical stimulation markers. This finding could also been seen in vivo in [^{18}F]FDG-PET/MRI, where the spleen was clearly visible after TLR stimulation and B cells showed upregulation of CD80 and CD86.
Conclusion: In vivo TLR stimulation can be visualized by increased [^{18}F]FDG uptake in lymphoid organs. The signal generated in the spleen after immunization might be mainly attributed to the activation of B cells within.
Advances in Knowledge and Implications for Patient Care: Knowledge of the composition of cells that take up [^{18}F]FDG during vaccination or in response to therapy may improve successful treatment of cancer patients in the future.

1. Introduction

PET is a highly sensitive, noninvasive detection method that provides three-dimensional information within the whole animal or patient. In contrast to optical imaging (bioluminescence, fluorescence) PET has no limit in imaging depth and offers the possibility to monitor changes in tumor metabolism and drug biodistribution. PET probes for cellular metabolic pathways enable quantitative in vivo measurements of cellular biochemistry. Clinically, the most common probe [^{18}F]-2-fluorodeoxyglucose ([^{18}F]FDG) is a positron-labeled glucose analog that accumulates in tissues with high rates of glycolytic metabolism and is commonly used to measure tumor cell glucose metabolism and its response to therapy [1].

High uptake of [^{18}F]FDG by inflammatory cells is a frequent cause of false positive results in [^{18}F]FDG-PET for cancer diagnostics [2,3], since it can be difficult to differentiate viable tumor cells from colocalized immune cells and to distinguish lymph node (LN) metastases from immune activation in reactive LN [4].

On the other hand, [^{18}F]FDG-PET has also gained an important role when imaging inflammatory processes in acute or chronic inflammation by [^{18}F]FDG-PET/computed tomography (CT) [5]. Shift in energy metabolism toward aerobic glycolysis is a hallmark of both, cancer and activated immune cells. For T-cells this switch from oxidative phosphorylation (OXPHOS) to aerobic glycolysis is a prerequisite for effector function as measured by cytokine (IFN-γ/Il-2) production. In contrast to effector function, T-cell proliferation and survival are independent of aerobic glycolysis and can be alternatively replaced by OXPHOS [6].

Immune stimulation is playing an important role in current and future therapies targeting the immune system to diseases like cancer.

Abbreviations: BCR, B cell receptor; CpG, cytosine-phosphate-guanine; CT, computer tomography; [^{18}F]FDG, [^{18}F]-2-fluorodeoxyglucose; LN, lymph node; LPS, lipopolysaccharide; MRI, magnetic resonance imaging; PET, positron emission tomography; TLR, toll-like receptor.

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When stimulatory immunological interventions such as vaccination (e.g., by nanoparticles) or immune checkpoint blockade are investigated, non-invasive PET imaging then might be capable for both, quantification of tumor response (at sites of tumor growth) and quantification of immune stimulation (at sites of lymphoid tissue). Thus, non-invasive monitoring of activation levels of the immune system by [^{18}F]FDG-PET/CT or [^{18}F]FDG-PET/MRI might develop to an important marker for clinical trials. In particular, in clinical trials when immune stimulation is not achieved in all patients this method might be reliable and broadly available to predict response to immunologic interventions at early time points. For suitable application of [^{18}F]FDG-PET in such a scenario, timing as well as magnitude of changes in energy metabolism has to be characterized.

Toll-like receptors (TLRs) are conserved pattern recognition receptors that respond to microbial components like lipopolysaccharide (LPS via TLR4) or unmethylated bacterial or viral CpG DNA (via TLR9). Besides the activation of innate immune responses TLRs play an important role in the activation of adaptive immune responses and in anticancer immunity [7–9]. While TLR1, TLR2, TLR4, TLR5 and TLR6 are predominantly expressed on the cell surface, TLR3, TLR7, TLR8, TLR9; TLR11, TLR12 and TLR13 are expressed in endosomal compartments [10–13]. Among these, TLR4 and TLR9 are the best studied TLRs and their ligands LPS and CpG DNA are used in this study to induce immune stimulation in vitro and in vivo.

TLR9 is mainly expressed by B cells and plasmacytoid dendritic cells (pDCs) [14,15]. For immunotherapeutic applications TLR9 is commonly activated by synthetic oligodeoxynucleotides (ODN) containing one or more CpG motifs. The development of CpG as vaccine adjuvant showed that by stimulating innate immunity TLR9 activation also enhances antigen-specific humoral and cellular immune responses. In mouse models CpG ODNs induce stronger Th1 immune responses than any other single vaccine adjuvant described [16,17]. Moreover, in humans CpG ODNs play a role in current vaccination approaches [18].

TLR4 is mainly expressed by macrophages, myeloid DCs, granulocytes and pDCs but also by (murine) B cells and T cells [19]. Upon activation B cells respond to LPS by proliferation, class switching, immunoglobulin secretion and plasmacytoid differentiation. In human B cells TLR4 expression has to be induced by specific stimuli, such as IL-4 [20].

Activation of T cells is achieved via their TCR complex by ligation with specific antigen or anti-CD3/anti-CD28 antibodies, causes proliferation of T cells and primes them for differentiation to effector subtypes. This process requires the coordinated production and surface expression of costimulatory molecules like CD25, CD44 and CD69 as well as the secretion of cytokines and chemokines [21].

In this study we used different TLRs in vitro and in vivo and anti-CD3/anti-CD28 antibodies in vitro to induce immune stimulation aiming on unraveling the role of immune cells influencing the strength of the [^{18}F]FDG signal during an immune response. Furthermore we wanted to elucidate which cells of the adaptive immune system account for the signal and to follow up the kinetics of the immune response via [^{18}F]FDG-PET imaging.

2. Material and methods

2.1. Animals

Male C57BL/6 J (Harlan Laboratories, Bicester, UK) mice were housed under specific pathogen-free conditions in the animal care facility in Mainz according to the guidelines of the regional animal care committee. All experiments were performed in accordance with federal guidelines and approved by the ethical committee of the state of Rheinland Pfalz (according to §8 Abs. 1 Tierschutzgesetz, Landesuntersuchungsamt; permission no. 23,177–07/G12-1-092). All in vivo imaging experiments were done under isoflurane anesthesia to minimize suffering.

2.2. Cell separation and stimulation

Primary immune cell populations (B cells, CD4$^+$ and CD8$^+$ T cells) were isolated from spleen cells by immunomagnetic depletion of lineage marker-deficient cell types using appropriate sorting kits (mouse B cell and CD4$^+$ or CD8$^+$ T Cell Isolation Kits (Miltenyi Biotec, Bergisch-Gladbach, Germany) following the manufacturers' instructions. Briefly, spleens were excised and single cell suspensions prepared by manual disintegration through a 40 μm nylon mesh. Then cells were incubated with biotin-antibody cocktails followed by anti-biotin microbeads and subsequent magnetic separation of the unlabeled cell fraction. Purity of enriched immune cells was controlled by flow cytometry using cell lineage markers, and typically exceeded 90%. In some experiments whole spleen cell suspensions were used in subsequent analysis.

3×10^5 cells/well were seeded into 48-well plates and stimulated with either Armenian Hamster anti-CD3 (145-2C11)/Syrian Hamster anti-28 (37.51) (1 μg/2 μg/ml) (Biolegend, London, UK), LPS (1 μg/ml) (Sigma, St. Louis, MO) or B-Class CpG ODN 1826 (0,5 μg/ml) (Invivogen, Toulouse, FR) over a period of 4 to 72 h at 37 °C in 5% CO_2.

2.3. In vitro [^{18}F]FDG uptake

Whole spleen cells or purified cell populations (B cells, CD4$^+$ and CD8$^+$ T cells) of naive C57BL/6 J mice were harvested after indicated time points of stimulation, washed with PBS and incubated with 370 kBq (PETNET, Erlangen, Germany) [^{18}F]FDG/well for 45 min at 37 °C in 5% CO_2. After two washes with PBS, counts per minute were measured using an automated gamma counter (Wizard^2 2470, PerkinElmer, Waltham, MA) and normalized to number of viable cells.

2.4. Micro-PET/MR imaging and ex vivo biodistribution

C57BL/6 J mice were immunized with LPS or CpG (0.2–10 μg/g body weight) over a period of 4, 48, 72 or 96 h. After fasting for 2 h, mice were injected intravenously with 7.7 ± 1.5 MBq [^{18}F]FDG followed by a 45 min uptake period. During the last 10 min of the uptake period mice were anesthetized with 2% isoflurane and MRI measurements (Material Map for coregistration of the PET scan; 3D Gradient Echo External Averaging (GRE-EXT), multi-field of view (FOV); slice thickness: 0.6 mm; TE: 2 ms; TR: 15 ms; flip angle: 25°) were performed followed by a 15 min static PET scan with the nanoScan PET/MRI (Mediso, Budapest, Hungary). Experiments were always done at the same time of day and mice were kept warm to reduce variations in blood glucose levels. PET data were reconstructed with Teratomo 3D (4 iterations, 6 subsets, voxel size 0.4 mm), coregistered to the MR and analyzed with pmod software (version 3.6). Ellipsoid ROIs were manually drawn to calculate the in vivo [^{18}F]FDG uptake in spleen vs. muscle as injected dose per milliliter (% ID/ml). For biodistribution studies organs were collected 1 h after activity administration, weighted and directly measured by an automated gamma counter (Wizard^2 2470, PerkinElmer) to calculate the percentage of accumulated activity as % ID/g.

2.5. Flow cytometry

For the ex vivo flow cytometric analysis of immunized mice, LN and spleens were excised and single cell suspensions prepared by manual disintegration through a 40 μm nylon mesh. Corresponding to in vitro [^{18}F]FDG uptake experiments, spleen cells and purified cell populations were also analyzed by flow cytometry for activation status. Cells were harvested, washed and stained with 0.4 μg/ml of the following antibodies for 15 min at 4 °C: rat anti-CD3-PE (17 A2), rat anti-CD4-FITC (GK1.5), rat anti-CD8-PE-Cy7 (53–6.7), Armenian hamster anti-CD11c-APC-Cy7 (N418), rat anti-CD19-APC-Cy7 (6D5), rat anti-CD25-APC-Cy7 (PC61), rat anti-CD40-APC (3/23), Armenian hamster anti-CD69-PaBlue (H1.2F3), Armenian hamster anti-CD80-FITC (16-10 A1),