Rheumatoid arthritis (RA) is the most common inflammatory joint disease, and early diagnosis is key for effective disease management. CD69 is one of the earliest cell surface markers seen at the surface of activated immune cells, and CD69 is upregulated in synovial tissue in patients with active RA. In this study, we evaluated the performance of a CD69-targeting PET agent, $[^{68}\text{Ga}]\text{Ga-DOTA-ZCAM241}$, for early disease detection in a model of inflammatory arthritis. Methods: A model of inflammatory arthritis was induced by transferring splenocytes from KRN T-cell receptor transgenic B6 mice into T-cell-deficient I-A$^\text{f2}$ major histocompatibility complex class II-expressing recipient mice. The mice were examined longitudinally by $[^{68}\text{Ga}]\text{Ga-DOTA-ZCAM241}$ PET/CT before and 3, 7, and 12 d after induction of arthritis. Disease progression was monitored by clinical parameters, including measuring body weight and scoring the swelling of the paws. The uptake of $[^{68}\text{Ga}]\text{Ga-DOTA-ZCAM241}$ in the paws was analyzed and expressed as SUV$_\text{mean}$. Tissue biopsy samples were analyzed for CD69 expression by flow cytometry or immunostaining for a histologic correlate. A second group of mice was examined by a non-binding, size-matched Affibody molecule as the control. Results: Clinical symptoms appeared 5–7 d after induction of arthritis. The uptake of $[^{68}\text{Ga}]\text{Ga-DOTA-ZCAM241}$ in the joints was negligible at baseline but increased gradually after disease induction. An elevated PET signal was found on day 3, before the appearance of clinical symptoms. The uptake of $[^{68}\text{Ga}]\text{Ga-DOTA-ZCAM241}$ correlated with the clinical score and disease severity. The presence of CD69-positive cells in the joints and lymph nodes was confirmed by flow cytometry and immunostaining. The uptake of the nonbinding tracer that was the negative control also increased gradually with disease progression, although to a lesser extent than with $[^{68}\text{Ga}]\text{Ga-DOTA-ZCAM241}$. Conclusion: The uptake of $[^{68}\text{Ga}]\text{Ga-DOTA-ZCAM241}$ in the inflamed joints preceded the clinical symptoms in the KRN T-cell transfer model of inflammatory arthritis, in accordance with immunostaining for CD69. $[^{68}\text{Ga}]\text{Ga-DOTA-ZCAM241}$ is thus a promising PET imaging marker of activated immune cells in tissue during RA onset. Key Words: rheumatoid arthritis; inflammatory arthritis; PET; CD69; inflammation

Received Jul. 12, 2023; revision accepted Oct. 10, 2023.

For correspondence or reprints, contact Fredrik Wermeling (fredrik.wermeling@ki.se) or Olof Eriksson (olof.ekriskon@ki.ki.se).

*Contributed equally to this work.
†Contributed equally to this work.
Published online Nov. 30, 2023.

COPYRIGHT © 2024 by the Society of Nuclear Medicine and Molecular Imaging.
used in clinical routine for detection and staging in the oncologic setting. However, its applications in RA and inflammatory diseases are not widespread, mainly because of the lack of PET probes selective for immune cells. The metabolic PET marker $[^{18}\text{F}]$FDG has been the most widely used so far for imaging of inflammation in RA patients (11,12). $[^{18}\text{F}]$FDG studies have demonstrated the detection and quantification of several types of arthritis activity and displayed uptake in fibroblasts, neutrophils, and macrophages when exposed to the inflammatory cytokines tumor necrosis factor and interleukin 1. However, almost no uptake in T cells has been observed, and in nonsevere and remission patients, the results have been inconclusive (11,12). As a general marker for glucose metabolism, the common limitations of $[^{18}\text{F}]$FDG include nonspecificity and false-positive findings in areas with high metabolic activity, such as the brain, heart, activated muscles, or brown adipose tissue and tumors.

Several other radiotracers, including those targeting macrophages, bone metabolism, vascular adhesion protein 1, angiogenesis, and cell proliferation, have been evaluated in RA (11–14). However, most of these radiotracers have been evaluated only in small patient cohorts or tested only preclinically and thus require further clinical evaluation (13–16). Thus, despite intense efforts in this area, there is an urgent need for PET imaging probes specific for noninvasive detection of immune cell activation in RA.

CD69 is an early activation antigen expressed by immune cells during activation. Limited expression is seen in peripheral blood leukocytes of healthy individuals. In contrast, increased expression is seen in T cells, B cells, and neutrophils in synovial tissue of RA patients, making it a promising target for studying aspects of the adaptive and innate immune reactions during RA onset and progression (17–20). Recently, radiolabeled antibodies directed toward CD69 were described and evaluated as imaging agents for detection of tumor-infiltrating activated immune cells in the oncologic context (21,22). However, antibodies are not ideal PET imaging agents because of their large size and slow clearance. Therefore, we have developed ZCAM241, a small protein-based Affibody (Affibody AB) molecule scaffold with nanomolar affinity for human and murine CD69 (23).

In this study, we examined the potential of $[^{68}\text{Ga}]$Ga-labeled ZCAM241 for early PET detection of activated immune cells in tissue in a mouse model of induced inflammatory arthritis.

**MATERIALS AND METHODS**

**Chemical Synthesis and Radiolabeling of Affibody Molecules**

ZCAM241 is an Affibody molecule selected for binding to the extra-cellular domain of human recombinant CD69 (Supplemental Table 1 [supplemental materials are available at http://jnm.snmmjournals.org]) (23,24). The chemical synthesis and the $[^{68}\text{Ga}]$Ga radiolabeling of DOTA-conjugated ZCAM241 and the nonbinding, size-matched Affibody molecule DOTA-ZAM106 as the control are described in detail in the supplemental materials.

**In Vitro and In Vivo Characterization of $[^{68}\text{Ga}]$Ga-DOTA-ZCAM241**

Unlabeled ZCAM241 has been evaluated in detail, such as with respect to affinity toward CD69 (23). DOTA-ZCAM241 labeled with $[^{111}\text{In}]$In has been studied for in vivo biodistribution and has demonstrated binding to, for example, activated human peripheral blood mononuclear cells (23). Here, we verified that radiolabeled $[^{68}\text{Ga}]$Ga-DOTA-ZCAM241 retained the biodistribution, stability, and binding of previously evaluated radiolabeled analogs (supplemental materials; Supplemental Figs. 1–3).

**Adaptive T-Cell Transfer and Joint Evaluation**

Experiments used 8- to 12-wk-old sex- and age-matched mice. KRN.B6 mice were generated and provided by Diane Mathis and Christophe Benoist (Harvard Medical School) (25). KRN.B6.CD45.1 mice were generated by crossing KRN.B6 mice with CD45.1 mice (stock number 002014, Jackson Laboratories). TCRb$^{-/-}$I-A<sup>b</sup>$/^{-/}$I-A<sup>Ag7/8</sup>$ mice were generated by crossing B6.TCRb mice (TCRB<sup>-/-</sup>I-A<sup>b</sup>$/^{-/}$I-A<sup>Ag7/8</sup>$) with nonobese diabetic mice (stock number 001976, Jackson Laboratories). Primers used for genotyping are detailed in the supplemental materials. To induce disease, KRN.B6 splenocytes were prepared by pressing the spleen through a 40-μm cell strainer with a 3-mL syringe plunger. Roughly 2 × 10<sup>6</sup> cells were injected via the tail vein into TCRb<sup>-/-</sup>I-A<sup>b</sup>$/^{-/}$I-A<sup>Ag7/8</sup>$ recipient mice. The severity of arthritis was scored every 2–3 d by clinical examination of each paw and ankle (0, no swelling; 3, maximal swelling), adding up to a total clinical score (0–12) per mouse. The weight of the animals was monitored every 2–3 d. At the end of each experiment, different organs and blood were collected for further analysis.

$[^{68}\text{Ga}]$Ga-DOTA-ZCAM241 PET/CT Imaging of Arthritic Mice

The animal experiments were authorized by the Animal Ethics Committee of the Swedish Animal Welfare Agency and performed according to institutional guidelines (Uppsala University Guidelines on Animal Experimentation, UFV 2007/724) and ARRIVE 2.0 guidelines.

The study design was a longitudinal imaging study to follow each mouse through 4 PET scans over 12 d, from before disease induction and during disease progression. The detailed ethical considerations and the in vivo study design are described in the supplemental materials.

Each mouse (5 female mice; weight, 20–23 g at the start of the study) was imaged by $[^{68}\text{Ga}]$Ga-DOTA-ZCAM241 (target dose of 2 MBq) PET 4 times: before (baseline) and then 3, 7, and 12 d after induction of inflammatory arthritis (Supplemental Fig. 1B). The mice were euthanized after the last PET scan, and biopsy samples were taken.

A control study was conducted separately with the nonbinding Affibody molecule $[^{68}\text{Ga}]$Ga-DOTA-ZAM106 using the same protocols for imaging, disease induction, and monitoring of clinical symptoms as described earlier for $[^{68}\text{Ga}]$Ga-DOTA-ZCAM241. Briefly, $[^{68}\text{Ga}]$Ga-DOTA-ZAM106 was evaluated by PET/CT (target injected dose, 2 MBq; same scanning protocol in 4 female mice; weight, 20–23 g at the start of the control study) at 4 time points (baseline and 3, 7, and 10 d after disease induction).

The PET scanning protocol, the image analysis methodology, and the histologic analysis of postmortem biopsy samples are described in detail in the supplemental materials.

**Flow Cytometry**

Single-cell suspensions from lymph organs were collected by pressing through a 40-μm cell strainer with a 3-mL syringe plunger. Single cells from the ankle joints of the hind paws were prepared by cutting joint tissue into small pieces in cold phosphate-buffered saline, vortexing, and filtering through a 40-μm cell strainer. Cells were filtered and stained with BioLegend’s CD19-Alexa647 (catalog number 115522), CD45.1-BV605 (catalog number 110738), CD45.2-BV785 (catalog number 109839), TCRβ-BV711 (catalog number 109243), and CD69-PE (catalog number 310905), as well as the Fixable Aqua Dead Cell Stain Kit (catalog number L34965; Invitrogen), for 30 min. After staining, cells were acquired using BD LS RW.Fortessa. Generated flow cytometry standard files were analyzed by FlowJo version 10 (FlowJo).
ZCAM241 in healthy animals demonstrated suitable biodistribution and uptake to baseline demonstrated a similar increase with time from injection of the model (Fig. 1A). Weight decreased 5 d after injection (Fig. 1B), reaching a maximum drop of approximately 10%. IgG anti-glucose phosphate isomerase (GPI) levels were significantly different between baseline and day 12 (Fig. 1C). The uptake in PET images was already visibly increasing on rear paws 3 d after injection (Fig. 2A) and in line with corresponding CD69 staining (Fig. 2B).

**RESULTS**

**Progression of Joint Disease**

The disease development of the mice was monitored by scoring the swelling of the paws, including the ankle, and measuring the weight of the animals. The clinical score started to increase 7 d after injection of the model (Fig. 1A). Weight decreased 5 d after injection (Fig. 1B), reaching a maximum drop of approximately 10%. IgG anti-glucose phosphate isomerase (GPI) levels were significantly different between baseline and day 12 (Fig. 1C). The uptake in PET images was already visibly increasing on rear paws 3 d after injection (Fig. 2A) and in line with corresponding CD69 staining (Fig. 2B).

[^68Ga]Ga-DOTA-ZCAM241 Uptake Increased During Progression of Inflammatory Arthritis

The in vitro and in vivo characterization of [^68Ga]Ga-DOTA-ZCAM241 in healthy animals demonstrated suitable biodistribution (Supplemental Figs. 2 and 3A–3C; details in supplemental materials) and retained binding toward CD69 (Supplemental Fig. 3D). These results guided the design of the imaging protocol in the arthritic mice.

The PET images were quantified by calculating the SUVmean of the rear joints. As seen visually in Figure 2, the SUV increased gradually and almost linearly from day 0 (SUVmean, 0.21 ± 0.04, n = 5) to day 3 (SUVmean, 0.69 ± 0.25, n = 5), day 7 (SUVmean, 1.06 ± 0.09, n = 5), and day 12 (SUVmean, 1.53 ± 0.12, n = 5; Fig. 3A). The SUVmean of each mouse is displayed in Figure 3B, where all animals followed similar uptake pattern. Most variation was noticeable at the day 3 time point, where 1 animal (mouse 2; Fig. 3B) exhibited a higher increase in joint uptake than the rest of the animals. The ratio of [^68Ga]Ga-DOTA-ZCAM241 uptake to baseline demonstrated a similar increase with time from induction of disease (Fig. 3C). In addition, the ratio of [^68Ga]Ga-DOTA-ZCAM241 uptake to baseline demonstrated a positive correlation with the clinical score of the joints (r = 0.82, P < 0.0001; Fig. 3D).

Uptake of the Control Nonbinding Peptide [^68Ga]Ga-DOTA-ZAM106 in Inflammatory Arthritis

The ratio of [^68Ga]Ga-DOTA-ZAM106 uptake to baseline also increased over time (Supplemental Fig. 4A), although to a lesser extent than for [^68Ga]Ga-DOTA-ZCAM241. The correlation between [^68Ga]Ga-DOTA-ZAM106 uptake ratio to baseline and clinical score of the joints indicated a positive connection (r = 0.87, P < 0.0001; Supplemental Fig. 4B) but was less pronounced than for [^68Ga]Ga-DOTA-ZCAM241. The disease progression in the mice being investigated with the control nonbinding peptide [^68Ga]Ga-DOTA-ZAM106 was similar to that monitored by clinical symptoms such as swelling of the paws and weight changes (Supplemental Figs. 4C and 4D). Histology of the joints demonstrated the presence of CD69-positive cells after euthanasia at the day 10 time point (Supplemental Fig. 4D).

Expression of CD69 in the Lymph Nodes Was Higher Than in the Joints

Besides analysis in the joints, CD69 expression levels were analyzed in different cell populations and organs (axillary, brachial, inguinal, mesenteric, and popliteal lymph nodes, as well as the spleen and joints) on day 12. The representative histogram gating is presented as viable singlets in Figure 4A, and quantification of the plots is presented in Figure 4B. The frequency of B cells and T cells in the CD69-positive viable singlet gate is shown in Figure 4C, and the quantification of plots is shown in Figure 4D. Fluorescence staining of the lymph nodes at baseline (Supplemental Fig. 4A) and 12 d after injection of arthritis induction (Supplemental Fig. 4B) demonstrated increased expression of CD69, in line with flow cytometry results. [^68Ga]Ga-DOTA-ZCAM241 uptake of the right (SUVmean, 3.6 ± 1.09) and left (SUVmean, 3.3 ± 0.72) axillary lymph nodes was strong 12 d after disease induction, higher than the background uptake in muscle (SUVmean, 0.45 ± 0.34; Fig. 4E) and consistent with the presence of CD69-positive cells seen by flow cytometry at the same time point.

**DISCUSSION**

Appropriate diagnosis and prediction of the disease progression of the individual RA patient are fundamental for successful

**FIGURE 1.** (A) Quantification of joint inflammation (clinical score, 0–12; 0–3 points per paw) as part of characterizing induced joint inflammation in KRN T-cell adoptive transfer model. (B) Weight change presented as percentage of weight at day 0. (C) Serum IgG anti-GPI levels comparing day 0 and day 12. *P < 0.01, by unpaired t test (n = 5). GPI = glucose phosphate isomerase; OD = optical density.

**FIGURE 2.** (A) PET images of [^68Ga]Ga-DOTA-ZCAM241 uptake at baseline and 3, 7, and 12 d after injection as in KRN T-cell adoptive transfer model. (B) CD69 immunofluorescence staining of joints of representative animals during matching time points.
[O]In this study, we wanted to assess PET tracer $[^{68}\text{Ga}]\text{Ga-DOTA-ZCAM241}$ uptake in a mouse model of inflammatory arthritis. The model we used is a KRN T-cell transfer model, in which splenic T cells are injected into T-cell–deficient mice. The KRN T-cell transfer model has been reported to be reproducible and show clinical signs of disease onset by day 7 after induction, with infiltration of macrophages and neutrophils into the joints, as well as cartilage damage and bone resorption. T cells have not been detected in the joints but have been reported to be present in the popliteal lymph nodes. Disease severity usually reaches a maximum peak after around 2 wk of induction. Similarly, in this study, we observed that the mice developed clinical symptoms in the form of swelling of the joints around day 7, but the average weight decreased a little earlier, on day 5. The uptake of $[^{68}\text{Ga}]\text{Ga-DOTA-ZCAM241}$ in the joints was already visually apparent on PET images 3 d after induction of the model, which is earlier than the clinical signs started appearing. The largest variability in SUV uptake between individual animals was also on day 3, which could refer to the variation in magnitude of the initial immune reaction. Otherwise, the animals followed approximately the same pattern of disease progression. $[^{68}\text{Ga}]\text{Ga-DOTA-ZCAM241}$ uptake gradually increased with time, which is consistent with the increasing severity of the inflammation and the correlation between SUV ratio and clinical score.

The uptake of $[^{68}\text{Ga}]\text{Ga-DOTA-ZCAM241}$ was low in or absent from the joints before disease induction (SUV$_\text{mean}$ $\sim$ 0.2; Fig. 3A), but it increased almost 10-fold 12 d after injection (SUV$_\text{mean}$ $\sim$1.5). The uptake of $[^{68}\text{Ga}]\text{Ga-DOTA-ZCAM241}$ in the lymph nodes on day 12 was even stronger, with SUV$_\text{mean}$ greater than 3 (Fig. 4E). However, because of the small size of the animal model compared with the resolution of the scanner, as well as the proximity of high focal uptake in the kidneys, it was difficult to measure the CD69 signal from the lymph nodes in vivo. Thus, the absolute values of the uptake of $[^{68}\text{Ga}]\text{Ga-DOTA-ZCAM241}$ in the lymph nodes should be considered with caution. Still, the relative uptake patterns are in agreement with the flow cytometry data, which indicate a higher proportion of CD69-positive immune cells in the lymph nodes than in the joints (Fig. 4B). In addition, flow cytometry analysis of the lymph nodes identified that the main CD69-expressing cells were B cells, followed by T cells. This is in line with the interaction of autoreactive B cells and T cells, resulting in IgG anti-GPI production that is central to this disease model.

Strong inflammation in tissue involves not only recruitment of immune cells but also increased perfusion and vascular permeability. The last 2 factors are known to potentially contribute to increased nonspecific PET tracer uptake in tissue. Thus, it is important to control for nonspecific uptake in sites of inflammation.

After analysis of $[^{68}\text{Ga}]\text{Ga-DOTA-ZCAM241}$, we proceeded to investigate in another batch of animals the uptake of the nonbinding Affibody molecule $[^{68}\text{Ga}]\text{Ga-DOTA-ZAM106}$ as a control, using the same experimental setup. The uptake of $[^{68}\text{Ga}]\text{Ga-DOTA-ZAM106}$ followed the same pattern by increasing in the joints with time, but it was lower than with $[^{68}\text{Ga}]\text{Ga-DOTA-ZCAM241}$. This indicates that there is a separate mechanism for nonspecific uptake, likely because of increased perfusion and permeability resulting from the inflammatory environment. Therefore, there is most likely a nonspecific component for the uptake of $[^{68}\text{Ga}]\text{Ga-DOTA-ZCAM241}$ in addition to binding to CD69-positive immune cells in tissue. However, the animal model of inflammatory arthritis used in this study exhibits stronger and more acute inflammation than the usually chronic and low-intensive process seen in RA in humans. Thus, it can be expected that the nonspecific uptake of $[^{68}\text{Ga}]\text{Ga-DOTA-ZCAM241}$ resulting from increased perfusion and vascular permeability would be less pronounced in patients.

FIGURE 3. $[^{68}\text{Ga}]\text{Ga-DOTA-ZCAM241}$ uptake expressed as SUV$_\text{mean}$ (each point represents average and SD of 5 scans) over time in joints either at group level (A) or in individuals (B). Error bars for day 0 time point in A are too small to be visualized. Asterisks indicate significance compared with baseline at day 0. (C) Uptake of $[^{68}\text{Ga}]\text{Ga-DOTA-ZCAM241}$ expressed as ratio to baseline in individuals. (D) Correlation of uptake of $[^{68}\text{Ga}]\text{Ga-DOTA-ZCAM241}$ in joints with clinical score. *** $P < 0.001$. **** $P < 0.0001$. M1–M5 = mouse 1 to mouse 5.
opted to use the nonbinding, size-matched Af
suitable blocking ligand different from the Af
mended for validation of PET tracers. Because of the lack of a
binding; thus, blocking with an unrelated binder is usually recom-
298
THE JOURNAL OF NUCLEAR MEDICINE

CD69-positive viable singlet gate. (D) Quanti-
3). (C) Representative plot showing frequency of B cells and T cells in
CD69-positive viable singlet gate (n = 3). (D) Quantification of plots in C, with per-
centage of B cells and T cells in CD69-positive viable singlet gate (n = 3). (E) SUV
mean uptake of left and right axillary lymph nodes and muscle at
day 12. ***P < 0.001, by ANOVA (n = 5). Axi = axillary; Bra = brachial;
Freq. = frequency; Ingu = inguinal; LN = lymph node; Mes = mesenteric;
Pop = popliteal; TCRβ = T-cell receptor β.

Increased uptake of the CD69-directed peptide [68Ga]Ga-
DOTA-ZCAM241 was seen in the paws of mice with induced
inflammatory arthritis, which preceded the appearance of clinical
symptoms. [68Ga]Ga-DOTA-ZCAM241 is thus a potential candidate
for PET imaging of activated immune cells during RA onset.

**DISCLOSURE**

The study was funded by JDRF (1-SRA-2020-973-S-B), the
Science for Life Laboratory, the Swedish Research Council (2020-
02312 to Olof Eriksson, 2019-05115 to John Löfbom, 2019-01415
to Olle Korsgren, and 2021-03178 to Fredrik Wermeling), the
Swedish Cancer Society (CAN 2017/649 and 20 1114 PfJ to John
Löfbom and 20 1114 PfJ and 22 0546 SIA to Fredrik Wermeling),
Vinnova (2019/00104 to John Löfbom), the China Scholarship
Council (to Yunbing Shen), ExoDiab, the Nordic Foundation,
Diabetesfonden, Diabetes Wellness, the Sten A.
Swedish Cancer Society (2019-649 SIA to Fredrik Wermeling),
€

**KEY POINTS**

**QUESTION:** Can activated immune cells be visualized in inflam-
flammatory arthritis using a CD69-targeting PET tracer?

**PERTINENT FINDINGS:** CD69-directed peptide [68Ga]Ga-DOTA-
ZCAM241 displayed increased uptake in the paws of mice with
induced inflammatory arthritis, which preceded the appearance of clinical
symptoms. [68Ga]Ga-DOTA-ZCAM241 accumulation in the
paws was strong and consistent with disease duration, whereas a
nonspecific control peptide demonstrated only low binding.

**IMPLICATIONS FOR PATIENT CARE:** [68Ga]Ga-DOTA-ZCAM241
is a potential candidate for clinical PET imaging of activated
immune cells in the joints during onset of, for example, RA.

**CONCLUSION**

Limitations of the current study include the lack of blocking
studies in a subset of animals. However, there is a lack of available
CD69-specific binders and inhibitors or endogenous ligands, and
before the discovery of ZCAM241, only monoclonal or polyclonal
antibodies toward CD69 were available. Thus, no ligands are suit-
able for in vivo blocking, except for the CD69-targeting Affibody
molecule ZCAM241. ZCAM241 may also block potential off-target
binding; thus, blocking with an unrelated binder is usually recom-
298
THE JOURNAL OF NUCLEAR MEDICINE

molecule

[]^{68}\text{Ga}}\text{Ga-DOTA-ZAM106 to estimate the nonspecific uptake, as is
often done in similar situations.

Another limitation is that the [^{68}\text{Ga}}\text{Ga-DOTA-ZCAM241 and
^{68}\text{Ga}}\text{Ga-DOTA-ZAM106 PET scans were performed on different
groups of animals, which may have different severity levels of
inflammatory arthritis. This was done to decrease the number of
PET scans and tracer injections to which each animal was sub-
jected. It was considered better for the study design to obtain tracer
up take at 4 time points in 2 groups than to obtain 2 scans using 2 trac-
ers (the active and the control nonbinding PET probes). However,
the selected model is known for its reproducibility, which was con-
{}
Korsgren are shareholders of Antaros Tracer AB. No other potential conflict of interest relevant to this article was reported.

ACKNOWLEDGMENTS

The Preclinical PET/MRI Platform, Sofie Ingvast, Athanasis Bitzios, and Bogdan Mitran are acknowledged for expert technical assistance.

REFERENCES