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Present status and future trends in molecular imaging of lymphocytes

Chiara Lauri, MD Michela Varani, BSc, PhD, Valeria Bentivoglio, BSc, Gabriela Capriotti, MD, PhD, and Alberto Signore, MD, PhD

Immune system is emerging as a crucial protagonist in a huge variety of oncologic and non-oncologic conditions including response to vaccines and viral infections (such as SARS-CoV-2). The increasing knowledge of molecular biology underlying these diseases allowed the identification of specific targets and the possibility to use tailored therapies against them. Immunotherapies and vaccines are, indeed, more and more used nowadays for treating infections, cancer and autoimmune diseases and, therefore, there is the need to identify, quantify and monitor immune cell trafficking before and after treatment. This approach will provide crucial information for therapy decision-making. Imaging of B and T-lymphocytes trafficking by using tailored radiopharmaceuticals proved to be a successful nuclear medicine tool. In this review, we will provide an overview of the state of art and future trends for "in vivo" imaging of lymphocyte trafficking and homing by mean of specific receptor-tailored radiopharmaceuticals.

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Introduction

In the past decades it has become clear that immune system has a crucial role in the development, progression and maintenance of a large variety of disorders, for example cancer, inflammatory or autoimmune (AI) conditions and infective diseases as recently demonstrated by the cytokine storm during SARS CoV-2 infection.[1-3] The increasing knowledge of molecular biology of the diseases allowed the identification of the main protagonists of a given physio-pathological process and the possibility to use specific therapies and vaccines against them. In recent years, indeed, we are assisting to a quick progress in technology and pharmacology fields, as demonstrated by the development of different immunotherapies that are, nowadays, largely used for treating cancer and AI diseases. Indeed, by targeting specific molecules, antigens and pathways that are involved in the development of the disease, it is now possible to efficiently treat the single patient and the single lesion, thus allowing a personalized treatment.

Nuclear Medicine Unit Department of Medical-Surgical Sciences and of Translational Medicine, Faculty of Medicine and Psychology, "Sapienza" University of Rome, Rome, Italy.

Address reprint requests to Chiara Lauri, MD, Nuclear Medicine Unit, University Hospital Sant'Andrea, 00189 Rome, Italy. E-mail: chiara.lauri@uniroma1.it

However, to reprogram immune cells of the host against cancer, for example by removing intrinsic immune suppression, may also trigger a cascade of events that can be dangerous for the patient. Therefore, there is the need of novel non-invasive tools that are able not only to provide a "histological" characterization of the disease, but also to monitor cellular trafficking during and after immunotherapies (ie, check point inhibitors), thus providing a prompt evaluation of therapeutic efficacy and side effects. At the moment, indeed, therapeutic efficacy is assessed by measuring tumor volume with conventional radiological imaging modalities, such as ultrasounds (US), computed tomography (CT) or magnetic resonance imaging (MRI), but this approach can be misleading. A tumor enlargement after therapy may be caused by either a real progression of the disease that is not responding to treatment, or by an enrichment of helpful immune cells within the tumor microenvironment that are playing a crucial role against cancer. This situation is called "pseudo-progression" and, despite the evidence of a lesion enlargement, it is a sign of a good response to treatment by mean of an increased immune cell infiltrate.[4] To differentiate a progression from a pseudo-progression is not possible with conventional radiological imaging that is based only on "dimensional" criteria and fails to provide an "in vivo" characterization of infiltrating immune cells. On the other hands, molecular imaging of

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immune cell trafficking may achieve such differentiation. Radiomics is also showing to be promising in characterizing tumor heterogeneity and predicting both prognosis and response to treatment.[5]

Amongst the different cells involved in tumor microenvironment as well as in many chronic inflammatory diseases, T-lymphocytes are one of the most investigated, since their role in regulation of immune system in these conditions, is well known. Targeting tumor infiltrating T lymphocytes (TILs) is, therefore, becoming a promising strategy for diagnostic, prognostic and therapeutic purposes. Nevertheless, this task cannot be easily accomplished "ex-vivo" by biopsy, for example, due to the fact that is an invasive procedure, not all the patients are suitable for a biopsy, not all the lesions are accessible and, most important, the biopsy is limited to a single point and that it does not provide a comprehensive spatial and temporal evaluation of the neighbouring areas that are closed to sampled point.

In the era of "precision medicine," we are quickly moving from these traditional methods towards molecular imaging that allows the "in vivo" characterization of biologic processes at the cellular and molecular level. These burgeoning field is radically revolutionising the way of conceiving a disease, demonstrating to be useful not only for accurately diagnose a disease by assessing a specific cellular or molecular target, but also for selecting candidates to immunotherapy and for therapy follow-up purposes.[3,6]

In this optic, Nuclear Medicine (NM) offers a plethora of specific single photon emission tomography (SPECT) or positron emission tomography (PET) radiopharmaceuticals and strategies able to non-invasively assess and monitor immune cell subtypes in many clinical indications.

In this review, we will provide a panoramic overview of the state of art and future trends for "in vivo" imaging of lymphocytes trafficking. We will not describe direct cell labelling techniques or reporter gene techniques, but will concentrate on targeting lymphocytes by using specific receptor-tailored radiopharmaceuticals (Fig. 1, Table 1).

Present status: published radiopharmaceuticals

The use of antibodies against specific immune cell populations, to monitor their trafficking in vivo, has been largely investigated in both preclinical and clinical settings.[7,8] T-cells and natural killer (NK) cells play a crucial anti-tumor cytotoxic effect therefore, they can serve as potential targets for assessing the response to a specific cancer immunotherapy.[9-11] This would be accomplished by using antibodies against CD2 and CD7, that are expressed in their surface. Nevertheless, the use of a probe against these two markers would not allow discriminating between NK and T-cells.

Mayer et al., in a preclinical study, compared two mouse-anti-human antibodies directed against CD2 and CD7, respectively with a control anti-CD3 clone reporting no impact on, in vitro, T cell proliferation or apoptotic properties for both whole anti-CD2 and anti-CD7 and for their respective fragments.[8] Moreover, Fab tracers were also able to detect the accumulation of anti-tumor T-cells transferred in a mice with induced acute leukemia, thus providing useful information on response to adoptive cell therapy (ACT). Nevertheless, the use of whole anti-CD2 antibodies resulted in a systemic T-cells depletion and in a reduced anti-tumoral activity, in vivo, thus again underlying that playing with the immune system can be problematic and requires a special attention to the possible side effects on immune system itself.

PET isotopes, for example ^{11}C and ^{18}F, have been tested for imaging NK trafficking in murine models. The use of ^{11}C-methyl iodide- labelled NKs in fibrosarcoma xenograft models allowed the quantification of the number of effector

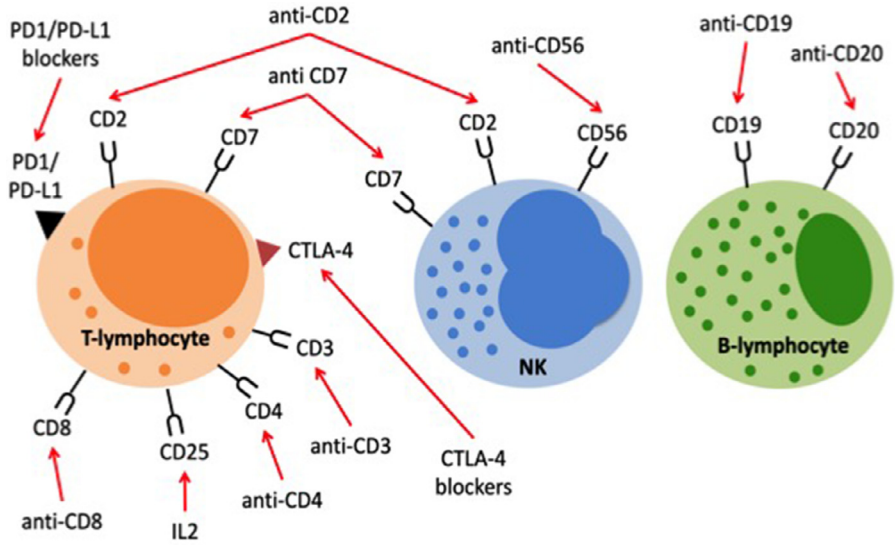

Figure 1 Schematic representation of targets for available receptor-tailored radiopharmaceuticals for imaging T-lymphocytes, NK cells and B-lymphocytes.

Table 1 SPECT and PET Radiopharmaceuticals for Imaging Immune Cells

Target	Probe	Immune Cell Population	Imaging Modality	Isotope	Applications	References
CD2	Anti-CD2	T-cells	SPECT	^{111}In	Preclinical	10, 14, 15
CD7	Anti-CD7	NK	PET	^{89}Zr ^{11}C ^{18}F	Preclinical	8, 12, 13
CD56	Anti-CD56	NK	SPECT	^{99m}Tc	Preclinical	9, 16
CD3	Anti-CD3 (Muromonab, Visilizumab)	T-cells	SPECT	^{99m}Tc	Preclinical Clinical	17-21
			PET	^{89}Zr	Preclinical	22-27
CD4	Anti-CD4	T-cells	SPECT	^{111}In	Preclinical	33
			PET	^{89}Zr	Preclinical	34
CD8	Anti-CD8	T-cells	PET	^{89}Zr ^{64}Cu	Preclinical	36, 37
CTLA-4	Anti- CTLA-4	T-cells	SPECT	^{111}In	Preclinical	39-41
PD-1/PD-L1	PD-1/PD-L1		PET	^{64}Cu ^{68}Ga ^{89}Zr	Preclinical	43, 45-49
CD25	IL2	T-cells	SPECT	^{123}I ^{99m}Tc	Preclinical Clinical	50-50, 62-69
			PET	^{18}F ^{68}Ga	Preclinical	70-73
CD20 CD19	Anti-CD20 (Rituximab, Ibritumomab)	B-cells	SPECT	^{111}In ^{99m}Tc	Clinical	76-80
	Anti-CD19		PET	^{124}I ^{89}Zr ^{64}Cu	Preclinical Clinical	81, 82-88
TNF-α	Anti-TNF-α (Infliximab)	B-cells	SPECT	^{99m}Tc	Clinical	89, 90
SDF1-α	CXCR4	T-cells B-cells Tumoral cells	SPECT PET	^{111}In ^{124}I ^{18}F ^{68}Ga	Preclinical Preclinical Clinical	99 100-104

cells within the tumor and provided information on their biodistribution in vivo.[12] Other researchers genetically modified NKs to express a chimeric antigen receptor that specifically binds to the tumor associated ErbB2 (HER2/neu) antigen and explored the ability of ^{18}F-FDG labelled NKs for imaging their trafficking in mouse sarcoma cell lines. Autoradiography showed an increased uptake of the radiopharmaceutical in HER2/neu positive tumors and histology demonstrated that genetically engineered cells accumulated in the tumors with higher extent compared to parental NK. Therefore, they concluded that this approach could be applied for monitoring NK-cell-based immunotherapies.[13] NKs has also been labelled with ^{111}In-oxine for SPECT imaging with gamma-camera in patients with metastatic renal carcinoma.[10] However, only a half of the metastatic lesions showed an increased uptake of radiolabeled NK cells and high amount of ^{111}In was released from the cells. Moreover, ^{111}In toxicity negatively impaired NK trafficking into the tumor. Similar findings were also achieved in patients with metastatic colorectal cancer and melanoma.[14,15]

In order to overcome the limitations of ex-vivo NKs labelling, it has been investigated the use of anti-CD56 monoclonal antibodies (mAbs) that bind to the CD56 expressed on NK surface. ^{99m}Tc-labelled anti-CD56 was administered in thyroid tumor xenografts, previously injected with human NKs. A control group of mice was injected with granulocytes. After 24 hours ^{99m}Tc-labelled anti-CD56 showed higher tumor-to-background (T/B) ratios in each tumor compared to the group that received granulocytes. T/B ratios also correlated with tumor infiltrating NKs at histology, thus concluding that radiolabelled anti-CD56 mAbs could represent a promising tool for non-invasively image NK cells before, during and after immunotherapies.[9,16] Nevertheless, more preclinical studies are needed to eventually confirm the feasibility of labelled anti-CD56 in imaging NK trafficking before translating this approach in humans.

CD3 is another marker of all T-cells that can be labelled with both SPECT and PET isotopes.

^{99m}Tc-labelled anti-CD3 has been used in several preclinical and clinical studies for imaging rheumatoid arthritis and acute rejection in renal transplants.[17-21] These studies mainly used Muromonab (OKT3), the first mAb that received the approval of FDA for therapy. But also ^{99m}Tc-Visilizumab, a humanized anti-CD3 mAb binding the T cell receptor (TCR) expressed on activated T cells homing in inflamed tissues, was used in different mouse

models demonstrating that unlabelled Visilizumab induces margination of peripheral blood mononuclear cells from circulation to small bowel and lymphoid tissues (lymph nodes, spleen, MALT tissue). This mechanism can be successfully visualized by ^{99m}Tc-Visilizumab.[17] Overall, these studies, demonstrated high uptake of the radiopharmaceutical in T lymphocytes infiltrates.[17-21]

Compared to SPECT radiopharmaceuticals, PET isotopes offer the advantage of higher T/B ratios and better image quality and several efforts have been devoted to the development of novel labelled CD3 mAb for PET imaging. In preclinical setting, ^{89}Zr-CD3 mAb was used to monitor T-cells trafficking in bladder and breast cancer and to assess the efficacy of anti CLA4 therapy in murine models bearing colon cancer.[22-24] In addition to tumoral uptake, these studies reported an increased uptake in spleen, thymus, lymph nodes, due to the high expression of CD3+ T-cells in these lymphatic organs, as well as in the liver, due to its role in the clearance of the mAb.[23, 25-27]

Nevertheless, given the affinity to native TCR, mAb against CD3 may have an effect on T-cells functions as demonstrated by Beckford and colleagues, who reported a relative depletion of CD4+ T-cells and an expansion of CD8+ T-cells after the administration of ^{89}Zr-anti CD3.[22]

Overall these studies, together with other studies using non radioactive probes for CD3+ cells imaging, for example using nanobubbles for US or nanoparticles for MRI imaging,[28-30] clearly demonstrated that this approach is very promising for visualizing and quantifying T-cells infiltrate in many oncologic and inflammatory diseases.

The approaches previously described allow imaging total T-cells population without the possibility to discriminate between regulatory T-cells, CD4+ and CD8+ since all these subsets express CD3, CD2 and CD7. Nevertheless, it is also possible to imaging CD4+ and CD8+ cells, separately. CD4+ T lymphocytes are involved in the pathogenesis of several immune-mediated diseases, such as multiple sclerosis and inflammatory bowel diseases (IBD) and other AI disorders.[31,32] ^{111}In-labelled anti CD4 (whole Ab) was used in animal models of colitis and showed increased uptake in inflamed bowel segments.[33] Others investigated the possible use of ^{89}Zr-anti CD4 cys-diabody, that does not include the Fc region of Abs, for imaging colitis in mice. An increased uptake in inflamed bowel as well as in regional lymph-nodes was observed as proof of the involvement of CD4+ cells in inflammatory conditions and this was also confirmed by immunohistochemistry. Moreover, they used unlabeled anti CD4 cys-diabody to assess its impact on the number of circulating T-cells and found that a dose of 40 μg is able to determine a transient depletion of T-cells in blood, lymph-nodes and spleen, thus suggesting an impaired functionality due to the binding of this diabody to TCR.[33,34]

The other major T-lymphocyte subset, namely the cytotoxic CD8+ cells, have been engineered and labeled with ß+ isotopes for immune-PET imaging.[35,36] Fragments and minibodies were, indeed, developed in order to accelerate plasma clearance, remove the Fc effector functions, thus being biologically inert but maintaining the specific targeting. These minibodies fragments have been labeled with ^{64}Cu[35] and ^{89}Zr[36] and were used in different preclinical models of diseases and immunotherapeutic models. In the first study of Tavaré et al., they injected two different ^{64}Cu labeled anti-CD8 minibodies into antigen-positive, antigen-negative, immunodeficient, antigen-blocked, and antigen-depleted mice in order to assess the distribution of the radiopharmaceuticals in lymphoid tissues. Both engineered minibodies retained their ability to target CD8+ cells in antigen-positive rats showing high uptake in spleen and lymph-nodes.[35] The same group also used ^{89}Zr for labelling anti-CD8 cys-diabody to detect changes in tumor-infiltrating CD8 cells in preclinical tumor and immunotherapy models including ACT transfer, agonistic antibody therapy, and immune-checkpoint inhibitors (anti−PD-L1), thus providing new tools to assess antitumor immune responses at different kind of immunotherapies.[36]

In recent years, immune checkpoint inhibitors have become a successful strategy to treat cancer. In particular, the discovery of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death protein 1 (PD-1) and its ligand (PD-L1), expressed on activated T-cells and showing inhibitory properties on T-cells activation, lead to the development of CTLA-4 and PD-1 blockers thus quickly becoming a promising immune-therapy for advanced cancer.[37] Nevertheless, a non-negligible percentage of patients fail to respond to these treatments, therefore, there was the clinical need to develop efficient tools to predict and to promptly evaluate patients' response. To this purpose, several strategies have been proposed to image both repressor and co-stimulatory molecules.[7]

In particular, antibodies specifically directed against PD-L1 have been labeled with ^{111}In, ^{64}Cu, ^{68}Ga and used in different tumor xenografts.[38-43] Overall, these preclinical studies found proportionality between radiopharmaceutical uptake and PD-L1 expression in tumoral cells, thus underlining the potential role of this approach to select patients eligible to anti PD-1/PD-L1 treatments. In addition to whole antibodies, ^{89}Zr labeled engineered fragments have also been developed and used for immuno-PET imaging in nude mice, bearing tumor xenografts with high PD-L1 expression, and in healthy non-human primates (NHPs), to assess the biodistribution.[44] The visualization of PD-L1+ tumoral cells in xenografts models appeared from 24 h after injection and persisted up to 120 h, with high T/B ratio. In the NHP models, PET imaging showed moderate accumulation in liver, kidneys, spleen, lymph nodes, and salivary glands.

Radiolabelled anti-PD-1 drugs have been also tested to monitor its expression on TILs. In melanoma models, ^{64}Cu-labelled anti-mouse PD-1 antibody showed high uptake in tumor lesions and spleen[45] and, in another experiment, ^{64}Cu-labelled humanized antibody pembrolizumab confirmed the feasibility of this approach in detecting PD-1 expression by a subpopulation of TILs.[46]

CTLA-4 has also become a target to predict a good response to immune checkpoint inhibitors and it has been mainly labelled with ^{64}Cu for PET imaging in preclinical setting.

In an interesting experiment, ^{64}Cu-labelled Ipilimumab was used in different cell lines of non-small cell lung cancers (NSCLC) showing different degrees of CTLA-4 expression. Notably, labelled Ipilimumab showed high uptake in A559 tumors (with high expression of CTLA-4) and lower uptake in H358 and A460 cell lines (with low expression of CTLA-4), thus once again underlying the potential role of these tailored approaches in quantifying target molecules and in providing crucial prognostic information in terms of therapy efficacy prediction.[47] Other authors, used ^{64}Cu labelled whole murine antibody against CTLA-4 in mice bearing tumor xenografts and in immune competent BALB/c mice in order to compare the biodistribution. PET images showed a significantly higher uptake in the first group, with a specific binding to CTLA-4, compared to normal mice.[48]

Imaging of CKs offers several advantages over the use of antibodies. First of all, CKs have a small molecular weight and therefore show a quick plasma clearance, secondly, they are widely available thanks to the large adoption of recombinant DNA methodologies, and finally they are not immunogenic. Amongst the different CKs that have been studied, IL-2 has become an attractive target for imaging in the last decades being a surrogate marker of activated T lymphocytes. This cytokine is composed by 133 amino acids and has a molecular weight of 15 KDa. It is produced and secreted by T-lymphocytes (mainly CD4 and CD8) and exerts a crucial role in promoting the proliferation of T and B lymphocytes, the differentiation of monocytes, macrophages, NK and oligodendrocytes. Biological effects of IL2 are mediated by its specific interaction with IL2 receptor (IL2R), a hetero-trimer composed by three units, namely CD25, CD122 and CD132, that is mainly expressed in activated T-lymphocytes. CD25 is the most important unit for biological activities of IL2. Therefore, imaging with radiolabelled IL2 provides an in vivo detection of CD25+ cells.

Our group has more than 30 years of experience in this field. The first attempts date back to the end of 80′s with ^{123}I-labelled IL2 that was successfully used, in both preclinical and clinical studies, for imaging different clinical indications characterized by T-lymphocytes infiltrate.[49-54] In particular, ^{123}I-IL2 was able to image pancreatic ß cells of rats bearing type 1 diabetes mellitus (DM1) thus, providing a marker of insulitis, that often precedes the clinical onset of DM1.[49,51] These findings were confirmed by CD25+ staining at histology that detected higher presence of activated T-lymphocytes in the pancreas of diabetic mice, compared to normal controls. This approach was also used in patients for detecting T-lymphocytes infiltrate in coeliac disease, prior and post gluten-free diet showing higher uptake of ^{123}I-IL2 in the bowel at basal time and lower uptake after an appropriate diet and scintigraphic findings were confirmed by jejunal biopsies.[52] In Crohn's disease (CD) ^{123}I-IL2 was able to differentiate quiescent disease from active phases.[53] Given the very promising results achieved also in other autoimmune and chronic inflammatory diseases, such as Hashimoto thyroiditis, Graves' diseases, vulnerable atherosclerotic plaques or rejecting transplants,[54-56] as well as oncologic diseases, for example head and neck carcinoma and hypernefroma,[57,58] several efforts have being devoted toward the development of ^{99m}Tc-labelled IL2.[59, 60] Preclinical and clinical studies on ^{99m}Tc-HYNIC-IL2 achieved similar results in terms of specific binding to IL2R, distribution and safety obtained with iodinated IL2 with the advantage of the use of an isotope that is less expensive, does not require cyclotron, is more available, has better physical and dosimetric properties and it is more suitable for gamma-camera imaging (Fig. 2). Overall preclinical and clinical studies with labelled-IL2 showed quick plasma clearance, high uptake in kidneys, liver and spleen. ^{99m}Tc-HYNIC-IL2 was largely used in the past decades for imaging the same indications previously studied with iodinated-IL2, with the addition of Sjögren's Syndrome and melanoma lesions.[61-68] In particular, in the most recent clinical study, scintigraphy with ^{99m}Tc-HYNIC-IL2 was performed at basal time and after immunotherapy with Ipilimumab and Pembrolizumab in five patients with metastatic melanoma. The uptake of labelled IL2 in cutaneous lesions was correlated with the entity of TILs at biopsy and, interestingly, more avid lesions showed a better response to treatment compared to lesions that showed lower or absent uptake at basal scintigraphy, thus underlying the potential role of this approach not only in selecting patients eligible to a specific immunotherapy, but also in discriminating true progression (bad prognosis) from pseudo-progression (good prognosis).[3,68]

More recently, several efforts have been devoted to develop a reproducible radiolabelling procedure with ^{18}F and ^{68}Ga for immune-PET imaging.[69-72] First attempts were conducted by Di Gialleonardo et al. by using ^{18}F and obtaining good results in terms of biological activity of IL2 and its binding to CD25+ cells,[69] nevertheless, this procedure is extremely long, laborious and expensive, it requires cyclotron and very high activities of ^{18}F for the labelling and it ends with limited doses of radiopharmaceutical that would be sufficient for one or two patients maximum.[69-71] The increasing availability of ^{68}Ge/^{68}Ga generators is moving the attention of the researcher to develop ^{68}Ga-labelled IL2 but the choice of chelator is the main issue since most of these agents require high temperatures for the conjugation, thus

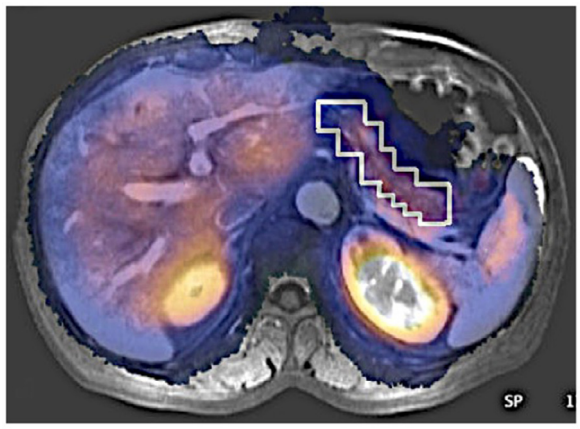

Figure 2 Axial sections of SPECT/MRI with ^{99m}Tc-HYNIC IL2 showing increased pancreatic uptake in a patient with insulitis.

causing degradation of IL2. Nevertheless, the advent of new chelating agents that can be handled at room temperature is opening new opportunities to label IL2 with ^{68}Ga whose half- life, perfectly fits with the short plasmatic half-life of IL2.

Finally, given the poor solubility in aqueous solvents and the tendency to aggregation of IL2, the labelling procedure is difficult but the promising and encouraging results achieved so far in many clinical indications are warranting the development of ready-to-use kits for labelling.

B-lymphocytes represent the other destiny of maturation and differentiation of a lymphocyte and they play a central role in humoral immunity of adaptive immune system, by producing antibodies after the binding of an antigen to B cell receptor (BCR9 expressed on their surface. Their role in autoimmunity, chronic inflammatory diseases and hematologic malignancies is nowadays well established. Indeed, in past decades several radiopharmaceuticals and approaches have been developed for tracking B-cells' network.[73] Direct labelling of lymphocytes has been attempted first with ^{51}Cr[74] and then with ^{111}In[75] and provided to be a non-invasive tool to in vivo monitor their homing in several AI diseases as well as in lymphoma. Nevertheless, a larger amount of literature exists on indirect labelling of B-cells, by using MAbs against specific surface antigens expressed by B-lymphocytes, in order to achieve a selective imaging of this population. Since the introduction of anti-CD20 mAbs in clinical practice for the treatment of lymphomas and several AI disorders, it has become a target for imaging with SPECT and PET radiopharmaceuticals.[76,77] Immunoscintigraphy with ^{99m}Tc-rituximab has been successfully used in patients with rheumatoid arthritis (RA), psoriasic arthritis (PsA), systemic lupus erythematosus (SLE), polychondritis, sarcoidosis, Behcet's disease, dermatopolymyositis, Sjogren syndrome (SS) studied by Malviya et al.[78]. In these patients, whole body planar images were acquired at six and 20 post injection (p.i.) before treatment with unlabelled rituximab (Fig. 3). The scan showed increased uptake of the radiopharmaceutical in affected sites, depending on the pathology, after 6 hours and persisting, with higher T/B ratios, at 20 hours. Interestingly, in patients with RA and PsA, ^{99m}Tc-rituximab showed a variable uptake in some affected joints but not in all the painful or swelling joints, thus underlying that different sites may show different degree of B-lymphocytes infiltration. This biomarker could be, therefore, extremely useful for mapping B-cells homing in the single patient, not only to assess the eligibility of that patient for the treatment with cold antibody, but also to select the sites that will benefit from this personalized treatment.[78,79] Rituximab has also been labelled with ^{124}I for immune-PET imaging in five patients affected by RA reaching similar conclusions and demonstrating once again the utility of labelled anti-CD20 in assessing disease activity, nevertheless the implementation of ^{124}I-rituximab in clinical practice is not justified by the high costs.[80] Relapsing or refractory non-Hodgkin lymphoma (NHL) is a paradigmatic example of the theranostic opportunities provided by NM imaging. A pre-therapy scintigraphy using ^{111}In- Ibritumomab (Zevalin), another anti-CD20 antibodies approved for the therapy of NHL, or in alternative a ^{89}Zr-rituximab immuno-PET, is performed in order to assess CD20 expression and to predict the response to cold Zevalin or to the radio-immunotherapy with ^{90}Y or ^{177}Lu-Zevalin.[81-86]

Anti-CD19 is also been labelled with ^{64}Cu for imaging experimental AI encephalomyelitis in mice. PET images showed significantly higher uptake in affected mice compared to normal controls as also demonstrated by ex vivo analysis.[87]

Anti-TNF-α mAbs represent another possible approach in in vivo image B-cell trafficking (Fig. 4).[3,11]

^{99m}Tc-infliximab scintigraphy in patients with CD was able to discriminate responders and non-responder patients treated with anti-TNF-α.[88] In another study conducted in patients with active RA ^{99m}Tc-infliximab scintigraphy was performed before intra-articular treatment with unlabelled infliximab, and provided variable degree of joint uptake being able to predict the success of therapy.[89]

Future trends

The rapid improvements in technology and radiochemistry are revolutionizing the approach to many oncologic and non-oncological diseases allowing an in vivo histological detection and quantification of a particular molecule, cell or pathway. PET radiopharmaceuticals such as ^{18}F, ^{68}Ga, ^{64}Cu are becoming more and more available in many centers and

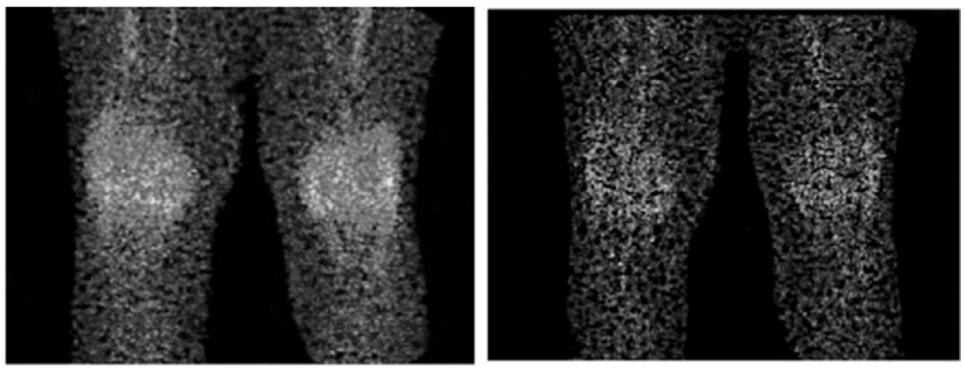

Figure 3 Anterior views of ^{99m}Tc-rituximab in a patient with rheumatoid arthritis before (left panel) and after therapy with 16nlabeled rituximab (right panel).

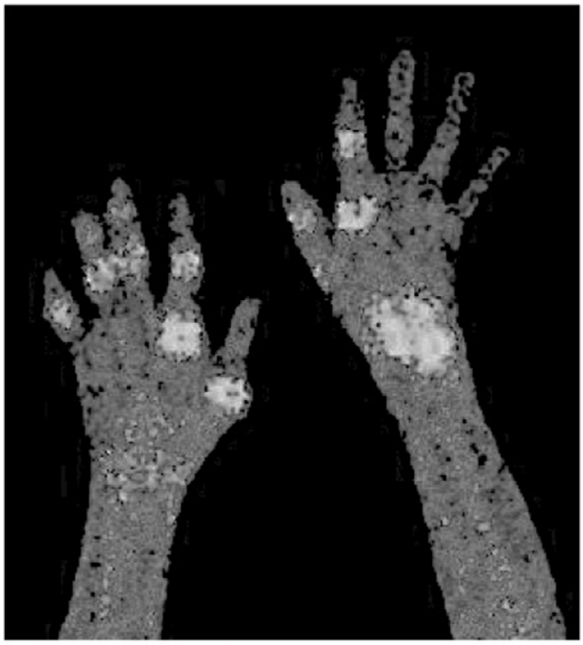

Figure 4 Anterior views of ^{99m}Tc-adalimumab (anti TNF-α) in a patient with rheumatoid arthritis showing increased expression of TNF-α in the hands.

may fit better with the biological half-life of small molecules, CKs and peptides. Indeed, despite the use of whole antibodies has been largely adopted, mainly in the past decades, their long circulation times due to slow plasma clearance and the need of long-lived isotopes remains a major concern that limits their use in clinical practice.[90] Therefore, CKs, peptides and minibodies will be the future of molecular imaging. The world of NM imaging of immune cells is constantly expanding and will continue to grow thanks to the discovery of new targets and the development of novel tailored radiopharmaceuticals able to image cancers, AI diseases, inflammatory and infective conditions at molecular level. What we can image today is just the "tip of the iceberg" but, in future, we will concentrate on other unexplored protagonists of tumor biology and other components involved in the pathogenesis of AI conditions or novel emerging infective diseases, thus providing new insights on their pathogenesis and new advanced tools for personalized diagnosis and therapy.

By using ^{18}F-FDG PET/CT, we recently speculated that lymphopenia observed during SARS CoV-2 could be due to the margination of T-lymphocytes into bowel rather their apoptosis or functional exhaustion, thus inducing large bowel inflammation.[91] Compared to normal subjects, COVID patients showed significantly higher FDG uptake into ileum, caecum and right colon wall. After recovery, this hypermetabolism tend to normalize in all these segments except for right colon. Moreover, we found an inverse correlation between CD4+ cells and ^{18}F-FDG uptake in the wall of large bowel, thus suggesting a possible migration of T-lymphocytes into the bowel and explaining the underlying causes of lymphopenia frequently observed in COVID patients. If confirmed by larger studies by using more specific strategies for imaging T-cells trafficking, this would be relevant to better understand the pathogenesis of this emerging infection and to develop specific therapeutic strategies or vaccines.

Imaging T and B-lymphocytes proved to be a successful strategy to evaluate, quantify and monitor their trafficking before and after immune-modulatory drugs providing undeniable prognostic information in terms of prediction of therapy response and, therefore, being able to plan the most appropriate treatment. Amongst the different approaches investigated, radiolabelled CKs, peptides and minibodies seem to be the most promising, versatile and potentially translatable in clinical practice for imaging a wide variety of immune-mediated diseases. At the moment, one of the major impediments is the lack of ready-to-use kits for labelling but several efforts have been already put in place and hopefully they will become available in upcoming years.

In addition to T and B-lymphocytes imaging, there are a multitude of attractive theranostic approaches under investigation and are providing very promising results such as fibroblast activation protein for imaging tumor stroma, angiogenetic molecules such as vascular endothelial grow factors, reporter genes therapies, CAR-T cells, the use of nanoparticles and many other alternative ways to image the complex interaction between immune system and host.[92] Amongst these novel approaches, imaging of chemokine receptor CXCR4, is becoming more and more attractive due to its role in hematopoiesis, organogenesis, vascularization and its involvement in several infective, inflammatory and neoplastic diseases.[93-97] CXCR4 is expressed by hematopoietic stem cells, as well as by T and B lymphocytes, neutrophils, monocytes, macrophages, eosinophils and in tumoral cells of both solid and hematological cancers. In particular, it is now well clear that, after its binding with its endogenous ligand, namely stromal cell-derived factor (SDF1-α), this cytokine triggers signal transduction cascades that induce tumor growth and survival. Moreover, the CXCR4 expressed by primary tumor, interacts with the SDF1-α expressed by distant organs, such as liver, bone marrow, lungs, thus promoting in distant metastatization.[95-97]

These aspects represent interesting opportunities for the development of CXCR4-specific probes for both imaging and therapeutic purposes and several efforts are being directed in this field. Both SPECT, mainly with ^{111}In[98] and PET radiopharmaceuticals, with ^{124}I and ^{18}F[99] have been developed and tested in preclinical models but the high liver and intestinal uptake and the low T/B ratio were the main limitations to their use in humans. One of the most promising radiopharmaceuticals for imaging CXCR4 network is represented by ^{68}Ga-Pentixafor that has been tested in preclinical models of human small cell lung cancer xenografts[100] as well as in humans mainly affected by lung cancer, pancreatic cancer, liver cancer and multiple myeloma, where ^{18}F-FDG has some limitations,[101,102] or for staging and therapy assessment of non FDG avid lymphoma variants.[103] Preliminary evidences suggest ^{68}Ga-Pentixafor as a valuable candidate, in future, for imaging CXCR4 network in cancer, being able to select patients who will benefit from target therapies and to evaluate treatment response with higher accuracy than conventional imaging modalities.

Overall, these novel molecular approaches could be of paramount importance also for the development of specific cancer vaccines able to induce immune response against specific tumor antigens. Many speculations and clinical trials are ongoing to assess the efficacy of cancer vaccines, nevertheless, despite encouraging results have been obtained in preclinical studies, their efficacy in clinical trials is controversial mainly due to the of the huge complexity in the targeting specific antigens that are exclusively expressed by cancer, thus preventing AI reactions against normal cells.[104] In this optic, NM imaging would facilitate the identification of specific target antigens thus accelerating the research on novel therapeutic strategies.

An important prerequisite for clinical translation of all these approaches is the need of prospective, randomized trials also better exploring dosimetric and genomic aspects in order to better plan a diagnostic and therapeutic strategy avoiding toxicity and undesirable effects.[105]

Conclusions

Medical research is moving fast and is quickly changing the way to approach to patients, diseases and treatments. There is now the clinical need to identify molecular targets that are specifically expressed by a pathologic condition for guiding a tailored therapy. But, it is interesting to note, that the concept of "personalized treatment" is rising up from the "depersonalization" of the patient that is no more conceived in his and/or her wholeness, rather than as a complex network of cells, antigens, pathways and biological phenomena that offer the possibility to be studied at molecular level. Nevertheless, identifying specific targets would allow choosing the most appropriate treatment for that patient and for that disease, thus providing crucial prognostic information for therapy decision-making.

Molecular imaging of lymphocytes is demonstrating to be a promising strategy to image a huge variety of diseases. Potentially, all the conditions in which immune system is involved, ranging from infective and inflammatory disorders, passing from AI diseased and ending to cancer, would benefit from this kind of approach. Many radiopharmaceuticals and diagnostic strategies have been, and are currently, investigated being the use of labelled CKs, peptides and minibodies the most promising. Several technical issues should be solved before their licit translation in clinical practice, but the quick progress in instrumentations, technology, radiochemistry and dosimetric field will make them valuable allies in our daily practice.

The future of molecular imaging is bright.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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