Introduction

Appearance of neutrophils and monocytes at the site of inflammation is a hallmark event during the inflammatory response (1). The recruitment of the different leukocyte subsets requires a coordinated interaction of a variety of molecules in a process known as the leukocyte recruitment cascade (2). Classical descriptions of this multistep-process consisted of leukocyte rolling, activation, adhesion and subsequent transmigration, involving cell adhesion molecules and chemokines as well as their respective receptors (3). Such steps were primarily identified by use of brightfield intravital microscopy. Owing to improvements in labelling of target structures, means of detection, and more elaborate surgical techniques, the recruitment cascade as defined above has been refined over the last years. The most intriguing new insights derived from studies utilising advanced optical imaging techniques are: (i) Implementation of intermediate steps: Use of time-lapse video microscopy revealed that following firm adhesion, leukocytes move slowly over the endothelial cell surface using their integrins Mac-1 and LFA-1 until they reach an area appropriate for transmigration (4, 5). (ii) Tissue-specific recruitment mechanisms: Much of the classical recruitment cascade has been studied by use of ex vivo flow chamber systems as well as in vivo model systems such as the cremaster muscle or the mesentery, which, however, may largely differ from recruitment mechanisms in other organs (6, 7). Employing improved surgical techniques and spinning disk confocal microscopy, McDonald et al. have shown that neutrophil accumulation in the liver is independent of selectins and β3-integrins, whereas CD44 and hyaluronan seem to hold crucial roles (8). In addition, application of (epi)fluorescence microscopy and two-photon microscopy to monitor neutrophil recruitment in carotid arteries and cremaster venules has allowed to identify the involvement of CCR1 and CCR5 in arterial but not venous recruitment (9). (iii) Leukocyte subset-specific mechanisms: Antibody labelling strategies and the use of transgenic mice with fluorescent leukocyte subsets (Table 1) have allowed to address differences in the recruitment between neutrophils and monocytes (10). (iv) Intercellular interactions: Leukocyte extravasation not just requires the cellular interaction between endothelium and the respective leukocyte subset, but also involves signals generated by cells situated in the extravascular space or circulating cells. In this context, deposition of chemotactic agents by activated platelets and neutrophils was shown to enhance adhesion and emigration of monocytes (11–14). By use of multi-channel fluorescence intravital microscopy Hidalgo et al. further showed the importance of interactions between neutrophils, platelets, and red blood cells in vascular occlusions in sickle cell disease (15).

Optical imaging techniques

The discovery and refinement of these leukocyte recruiting events was primarily achieved by the employment of intravital imaging methods. Some of these methods were already applied over 100 years ago, but recent advances allowed for improvements with regard to labelling strategies, speed, sensitivity, resolution, and tissue penetration. In this review we highlight the principles of intravital imaging of leukocyte recruitment by means of optical imaging techniques and emphasise the translation thereof into tissue-specific recruitment to the lungs, the liver and large arteries.

Keywords

Imaging, leukocyte trafficking / recruitment, cell migration, inflammation
imaging of leukocyte recruitment by use of fluorescence microscopy. Much of the review will be focused on intravascular processes. For in-depth revisions on phagocyte migration we would instead like to point some recent reviews (16, 17). Furthermore, we consider experimental perspectives for intravital microscopic imaging of inflammation. Despite their importance to the current knowledge and clinical applicability, modern, non-optical imaging techniques such as positron emission tomography, magnetic resonance imaging, (micro) computer tomography, optical imaging techniques such as positron emission tomography, (micro) computer tomography, or ultrasound will be neglected because of their lack of subcellular resolution (18, 19). Exciting optical modalities such as optical coherence tomography (OCT) (20, 21), photo acoustic tomography (PAI) (22), or coherence anti-stokes Raman scattering (CARS) (23) offer higher (PAI) or even subcellular resolution. Based on the principle of fluorescence, fluorescence microscopy (WF) has emerged as a widely used optical microscope modality because of its high selectivity and specificity, contrast usage was initiated in biological studies to selectively stain biological samples with fluorophores. Since then, (widefield) fluorescence microscopy (WF) has emerged as a widely used optical microscope modality because of its high selectivity and specificity, as well as enhanced contrast. Based on the principle of fluorescence, novel microscope modalities have been developed such as confocal

<table>
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<tr>
<th>Strain name</th>
<th>Fluorescent cell type</th>
<th>Reference</th>
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<tr>
<td>c-fms-gfp</td>
<td>GFP in mononuclear phagocytes</td>
<td>[103]</td>
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<tr>
<td>Cx3cr1-gfp</td>
<td>GFP expression correlates with Cx3cr1 transcription. Inflammatory monocytes are GFP&lt;sup&gt;hi&lt;/sup&gt;, resident monocytes are GFP&lt;sup&gt;lo&lt;/sup&gt;, NK cell subsets, microglia, and DCs are GFP&lt;sup&gt;int&lt;/sup&gt;</td>
<td>[104]</td>
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<tr>
<td>Lysm-gfp</td>
<td>GFP expression correlates with the transcription of lysozyme M. Circulating neutrophils are GFP&lt;sup&gt;hi&lt;/sup&gt;, while monocytes are GFP&lt;sup&gt;lo&lt;/sup&gt;.</td>
<td>[105]</td>
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<tr>
<td>MhcII-gfp</td>
<td>DCs are GFP&lt;sup&gt;hi&lt;/sup&gt;, B cells are GFP&lt;sup&gt;int&lt;/sup&gt;, and macrophages are GFP&lt;sup&gt;lo&lt;/sup&gt;</td>
<td>[106]</td>
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<td>Tie2-gfp</td>
<td>In the bone marrow ECs, HCs, and a small population of myeloid cells are GFP&lt;sup&gt;lo&lt;/sup&gt;. In peripheral blood, a small fraction of myeloid cells is GFP&lt;sup&gt;hi&lt;/sup&gt;.</td>
<td>[107]</td>
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<tr>
<td>Cd11c-yfp</td>
<td>YFP expression in DCs, monocyte subsets, NK cell subsets, and subsets of T cells.</td>
<td>[108]</td>
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<tr>
<td>Mrp8-gfp</td>
<td>GFP expression restricted to neutrophils</td>
<td>[109]</td>
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achievable depth discrimination (axial resolution) (32, 33) or classical laser scanning systems equipped with small diameter objectives employing the gradient index micro lens technology (GRIN lenses) (34), reduce the invasiveness of surgical manipulation. As a consequence, they potentially enable studying organs such as the liver in an even more physiological environment without significant loss of optical resolution. Application of optical fibres for two-photon microscopy is technically challenging due to difficulties in guiding a femtosecond pulsed laser through an optical fibre, the limited working distance of the fibres (and as a consequence limitations in imaging deep in organs) and reduced lateral and axial resolution. Even though the latter results in limitations in three-dimensional imaging, usage of fibre optics for two-photon microscopy in combination with GRIN lens technology has already been successfully applied for clinical in vivo studies of the skin (30). Moreover application of (compound) GRIN lenses for two-photon microscopy already yielded promising results for deep tissue brain and kidney imaging in vivo (34).

**Labelling target structures**

Early intravital microscopy studies analysing myeloid cell extravasation used brightfield microscopy. Such techniques were typically employed in tissues that were thin enough to allow white light transmittance through the sample. Classical intravital models which are still in use are the mesentery and cremaster muscle of small rodents. Although brightfield intravital microscopy is cheap and easily realisable, the major shortcoming is the lack of discrimination between cell subsets involved in leukocyte recruitment cascade. Hence, epifluorescence intravital microscopy has been introduced that overcomes this shortcoming by various approaches. Intravenous injection (i.v.) of rhodamine-6G or acridine orange, which accumulate in mitochondria or bind to DNA, respectively, allow for discrimination of nucleated (leukocytes) and non-nucleated (erythrocytes, platelets). These labelling methods have frequently been used in various models of leukocyte tracking. However, with increasing knowledge of the diversity of phagocyte subsets, these approaches have proven unsatisfactory and more specific labelling techniques have been developed. The adoptive transfer of purified cells from donor mice is one approach. Phagocyte subsets can easily be isolated by density gradients or FACS sorting from murine blood or bone marrow samples. Fluorescent labelling and subsequent i.v. injection allows for cell tracking in peripheral tissues. One has to keep in mind that a large number of cells get stuck in e.g. lung capillaries upon i.v. instillation, so that intra-arterial injection upstream of the tissue under observation may be preferable. Yet another pitfall is that isolated cells may be activated and thus affect their recruitment. Hence, it is critical to monitor activation (shedding of L-selectin, upregulation of CD11b) and apoptosis before cell injection. Alternatively, one may employ cell lines. However, considering their immaturity and their tendency to plug capillaries due to their large sizes, their use should be limited to rare cases where e.g. large cell numbers are needed.

The use of transgenic mice expressing fluorescent proteins in a cell-specific fashion is a more elegant method for specific cell labelling and is widely accepted to track individual leukocyte subsets. A variety of strains has been created to allow for tracking of myeloid cell populations in vivo (Table 1). However, care has to be taken when using mice where the gene encoding for a fluorescent protein has been knocked into the locus of a gene involved in recruitment. This is the case for Cx3cr1-egfp mice, where homozygous mice lack Cx3CR1 and thus recruitment of monocyte subsets may be affected (35). On the other hand, the clear advantages of fluorescent protein-encoding mouse strains over adoptive transfer approaches is that the target cells are not subjected to ex vivo handling and that the fluorescent tags do not become diluted or fade in dividing cells.

In recent years, the use of mice expressing fluorescent proteins has been complemented by the application of fluorescent antibodies towards myeloid cell subsets. The injection of antibodies to Gr1 and CD115 or F4/80 allows for labelling neutrophils or monocytes (36) independently of the genetic background of the mice (Fig. 2) Hence, this approach can easily be applied in any mouse strain. However, one has to bear in mind that some antibodies may affect leukocyte behaviour. In the case of antibodies to Gr1 it has been shown that higher doses of this antibody induce depletion of neutrophils, so that low concentrations have to be used to avoid such depletion (13, 37). An alternative antibody-based approach is the conjugation of antibodies to fluorescent beads (1 μm in diam-
Model systems to study leukocyte recruitment

Extravasation of myeloid cells is a crucial event during the inflammatory process. The understanding of the stepwise cascade has lead to the development of antagonists, of which an antibody to VLA4 (natalizumab) is in clinical use for multiple sclerosis and Crohn’s disease, while an antibody to LFA1 (efalizumab) intended for use in psoriasis has been suspended from the market due to the development of progressive multifocal leukoencephalopathy (2, 41). However, deeper understanding may allow for development of more specific inhibitors for custom-made therapies with reduced side effects.

The extravasation of myeloid cells in inflammation normally takes place in postcapillary venules of 15 to 50 μm in diameter. Several recruitment models have been introduced to study leukocyte recruitment in the microcirculation. Most models are acute preparations, whereof the mesentery or the cremaster are the most popular models. One advantage of these models is that the transparency of the tissue and the low movement artifacts allow for recording of extravascular leukocyte migration even for longer time periods (42–44). Also, investigations of endothelial permeability by use of fluorescent plasma tracers are permitted in these models. In addition, the preparation of cremaster and mesentery is fast and allows for stable preparations, so that movement artifacts only play a minor role in these models. However, the surgical intervention requires anaesthesia and causes trauma, thus increasing leukocyte-endothelial interactions (45, 46). Moreover, care has to be taken when choosing the model, as the various models exhibit important differences in haemodynamics and cellular composition. The cremaster muscle, for example, contains only very few mast cells while the mesentery is relatively rich in mast cells (47). Thus, the cremaster muscle is of limited suitability to study mast cell-dependent inflammatory recruitment. These models, however, bear the disadvantage that they include an acute surgical trauma, and that all studies can only be performed in the anaesthetised but not in the awake animal. Furthermore, these acute preparations are restricted to observation time and are not suitable for repeated assessment over time periods of days or even weeks. To overcome these drawbacks of acute microcirculation models, a variety of different chamber techniques have been introduced, of which dorsal skinfold chamber models are the most popular ones. In these long-term models intravital microscopy can be performed over a time period of several weeks. Applications range from ischaemia/reperfusion and healing of skin flaps, to angiogenesis, permeability, microcirculation, and transplantation of cells and tissues such as pancreatic islands (48), ovarian follicles (49), or tumour cells (50). The major advantage of the dorsal skinfold preparation over other microcirculatory models is that the model allows for repeated observations in unanaesthetised animals. The latter is important, as several gaseous anaesthetics were shown to interfere with leukocyte recruitment. Furthermore, if a recovery period of 2–3 days following preparation is provided, the effects of surgery-induced artifacts can be excluded. While the striated muscle of the skinfold preparation displays all segments of the microvasculature, i.e. terminal arterioles, nutritive capillaries, and postcapillary and collecting venules, lymphatic vessels cannot be detected. Disadvantages of this model are that the open wound may induce granulation tissue and inflammatory signals. In addition, the cover glass allowing direct observation creates an artificial surface that may alter cell activities such as motility. Finally, blood flow has to be monitored carefully, as too much tension exerted by the skin chamber may result in inadequate blood flow. Due to the limited field of view, observations made by intravital microscopy may sometimes be biased by the choice of region of interest. Hence, it is advisable to also employ recruitment models that are independent of imaging. Such models classically involve the assessment of leukocytic infiltrates into cavities or organs by flow cytometry. The analysis of leukocyte infiltration into cavities such as the pleura, the peritoneum, the lung, or subcutaneous air pouches requires lavaging, antibody labelling and subsequent FACS analysis (51, 52).

Recording leukocyte recruitment in solid organs

The above mentioned preparations remain models as under normal conditions inflammatory responses in these tissues are rather rare. In addition, evidence is accumulating that leukocyte recruitment may largely differ from the recruitment patterns observed in these model systems (6, 7). Thus, in recent years protocols were established that allow to directly studying vascular beds of solid organs, and consequently allow studying organ-specific recruitment patterns. In the following, we focus on imaging of lung, liver, and skin microcirculation as well as large arteries. Protocols are also available for other organs and tissues, such as lymph nodes, and bone marrow, (55–59), but are here neglected due to space limitations. Instead we would like to refer the reader to specialised reviews on these organs (60, 61).

Lung

The mechanisms of neutrophil recruitment into the lung remain ill-defined. Leukocyte recruitment into the lung is influenced by...
several factors including neutrophil deformability, adhesion molecules, and the unique capillary structure of the lung (62). On the way through the small pulmonary capillaries, neutrophils have to stop several times in order to change their shape and subsequently squeeze through the small vessels. Hence, the specific architecture of the lung brings about a prolonged transit time of the neutrophil through the lung and accumulation of neutrophil located primarily within alveolar capillaries. Inflammatory stimuli, primarily those which bind to seven transmembrane-spanning G protein linked receptors, induce changes in the cytoskeleton of neutrophils that reduce the capability to deform. The specific role of adhesion molecules and chemokines in neutrophil recruitment to inflamed lungs is not fully understood and seems to be context-dependent. While selectins seem to be of minor importance in LPS-induced lung injury, they hold prominent roles in complement-mediated lung damage models (63, 64). Similarly, the involvement of β₃-integrins in neutrophil recruitment to the lung depends on the animal model employed (65, 66). These data suggest that neutrophil recruitment into the lung is different from other vascular beds and intravital microscopic approaches may allow dissecting these mechanisms in detail.

Opposed to model systems described above, intravital microscopy of the lung is hampered by its complex optical accessibility and the severe respiratory movement artefacts. While protocols have been developed for larger animals such as dogs, rabbits or rats, such has proven difficult for mice. Intravital microscopy of lung microcirculation in larger animals typically relies on implantation of steel windows into the thoracic wall. Motional artefacts are counteracted by applying negative pressure. An adapted version of this approach has been transferred to mice (67), but only few applications were reported. The group of Kuebler et al. chose a different approach (68) by directly sealing the excised window with a transparent membrane. Coupling of the lung surface to the membrane was achieved by removal of intrathoracic air via a transdiaphragmatic intrapleural catheter, and lungs were allowed to move freely during the respiratory cycle. Direct contact of the lung surface with the cut edges of the partially removed ribs is prevented by the intermediate layer of parietal pleura and subpleural fascia, and neither structural nor functional defects in the subpleural area of observation were detectable. However, a potential limitation of this preparation is the restriction of the area of observation to the surface of the lung microcirculation where the vascular network is less dense as compared to the more central lung microcirculation (69).

This limitation may be overcome by a recently published protocol employing a fast, resonant-scanning based two-photon platform that allows for imaging up to 125 μm below the pleural surface with close to video rate acquisition speeds (70). A further advantage of this setup is the stabilised imaging that was achieved by use of a vacuum chamber placed on a thoracic window, whereby the lung was gently immobilised throughout the respiratory cycle. By positive pressure ventilation and image integration stable imaging over time was achieved. Another approach that may lead to reduction of the impact of respiration movement on the image quality, is utilisation of timed image acquisition (71). The latter approaches may ultimately enable tracking lung phagocyte movement.

Liver

Our current understanding of the basic function of the liver microcirculation, including specific microvessel responses to vasoactive substances, is mainly derived from studies that utilised transillumination intravital microscopy of the edge of a lobe of the mouse or rat liver (72). Transillumination of the edge of the liver provides sufficient signal and contrast to obtain images for accurate measurement of various parameters such as identification of individual inflammatory cell types and their contribution to modulating vascular function. However, since microcirculation is dissimilar from other parts of the liver, the edge of the liver is not ideal for intravital microscopy (73). Moreover, normal transillumination microscopy lacks the specificity and sensitivity in comparison with fluorescence based optical imaging.

For this reason, intravital microscopy of the liver is often performed with (epi-) fluorescence methods. In combination with appropriate fluorescent markers for specific labelling of leukocyte (sub-) populations or transgenic mouse models, visualisation of the microcirculatory system and cell recruitment can be achieved: Improved methods to provoke inflammation (ischaemia/reperfusion [74], obstructive cholesterasis [75], lipopolysaccharide challenge [76] or induction of sepsis [77]) further progressed the field, allowing studying of cell recruitment under different inflammatory condition. Fine tuning of tissue preparation models and surgery where one of the liver lobes is exposed, protected from air, and positioned on a stable surface resulted in reduced influence of motion and thus an increase in image quality even at locations deeper inside the liver. Moreover, since it became clear that baseline cell recruitment due to surgical manipulation in the liver is not significant (78), prolonged imaging of cell recruitment is feasible, allowing improved optimisation of the acquisition settings while still obtaining physiologically relevant findings. Application of more advanced optical modalities such as high-speed spinning disk confocal microscopy (7, 8, 79) provided improved depth penetration and axial resolution which enables imaging of cell recruitment in up to four dimensions deep in the liver. Moreover, simultaneous detection of multiple fluorophores is simplified in confocal-like systems, which often use multiple photomultiplier tubes (PMTs) to detect light of different wavelength in separate channels. Even though penetration depth is also slightly improved with spinning disk confocal microscopy it does not match the imaging depths as achieved with two-photon microscopy. Strangely enough, application of two-photon microscopy for studying cell recruitment in the liver is not often applied yet, even though its application provides several theoretical advantages such as increased depth penetration (currently = 80–100 μm in liver) and uncomplicated detection of multiple fluorophores. This is probably due to the relatively slow acquisition rates that until recently were achieved with two-photon microscopic systems at sufficient resolution. The current (commercially available) two-photon systems, however, do allow fast image acquisition at video rate with good resolution and could offer new opportunities for studying dynamic processes at deeper locations in the liver.
Skin

The skin is the organ most frequently exposed to injury and hence an intact immune response involving leukocyte extravasation is crucial. Among many other cell types, skin contains resident leukocyte subsets including Langerhans cells and γδ T cells, which serve as sentinels of pathogen invasion and innate-like effectors, respectively. Imaging immune responses in the skin has most often been done in the dermis of the ear because of its ease of access and relative natural transparency. The inner surface of the ear pinna of the mouse is an ideal site for intravital microscopy of immune responses in the skin because it has few hair follicles, allowing direct imaging of tissue without surgery. Blood vessels and cellular elements can easily be visualised owing to the thin epidermis (80, 81). A recent intravital microscopy study in the dermis of the ear pinna revealed a monocyte subset crawling along the endothelium of postcapillary venules which can quickly respond to injury and infection (82). Such responses were found to require the integrin LFA-1 and the chemokine receptor CX3CR1. Furthermore, 2-photon microscopy has allowed tracking of Leishmania major after inoculation into the skin (83). Following sand fly bites of mouse ears, neutrophils rapidly infiltrated and phagocytosed parasites. Once neutrophils underwent cell death they released parasites which were then taken up by macrophages. Alternatively, macrophages phagocytosed Leishmania-containing apoptotic neutrophils.

Large blood vessels

Atherosclerosis is a chronic disease of the large arteries where both innate (atherogenesis) and adaptive (progression and destabilisation) immunity responses are involved (84–86). The general idea of luminal cell recruitment by the vessel wall leading to atherogenesis, plaque progression, and plaque destabilisation is extrapolated from in vitro, histological or microcirculatory intravital studies. Moreover, the few studies utilising classical intravital imaging of exposed large arteries (9, 87–91), allow for easy quantification of luminal cell recruitment but lack the image quality required to study the process of phagocyte recruitment in more detail (Fig. 3). An important reason for the reduced quality is that intravital imaging of leukocyte recruitment in the large atherosclerotic prone arteries is strongly hampered by the thick arterial wall reducing light penetration and thus axial resolution, especially at deeper positioned structures of the artery. Due to poor axial resolution of “classical” intravital microscopy, it is difficult to discriminate between luminal and adventitial cell recruitment. Moreover, the movements of the arterial wall due to blood flow and respiration movement cause severe motional artifacts in the resulting images which further limit the applicability of classical intravital microscopy. Two-photon laser scanning microscopy (TPLSM) overcomes the limited penetration depth of other fluorescence techniques and has demonstrated its usefulness for structural and functional imaging of intact large arteries (92–96). Yet, for quantification of cell recruitment in vivo TPLSM is more difficult to because of its high axial resolution (and thus reduced overview in depth) and, to a lesser extend, the often smaller field of view (due to the frequently applied high magnification/numerical aperture objectives). Thus far, application of TPLSM was only utilised in few laboratories (9, 92, 97), mainly because of its sensitivity for sample movement. Due to its “pixel-by-pixel” scanned image acquisition, it suffers severely from the motional artifacts. Attempts have been made to overcome these limitations, either by (i) stabilisation of the artery, (ii) by exact timing of the acquisition of an image when non-motional period or (iii) by post image processing. (i) Stabilisation of the artery by embedding the surgically exposed carotid artery in stiffened material such as 1–3% agarose in buffer, does not provide enough stability to enable tracking of inflammatory cell recruitment (own observations). A more harsh method to stabilise the artery is to position a shovel-like plate underneath an exposed carotid artery (97). By gently lifting the plate, and thus the artery, stable in vivo imaging of the cells and structures of the artery...
Intravital imaging of inflammation

Stoletov et al. demonstrated that zebrafish fed a high-cholesterol diet exhibit increased plasma low-density lipoprotein and very low-density lipoprotein levels and increased lipoprotein oxidation, resulting in fatty streak formation resembling the process of atherosclerosis in humans (100–102). In zebrafish the process of atherogenesis can be followed over time and function of vascular cells can be studied in detail in vivo, without many of the imaging difficulties experienced in the various mouse models of atherosclerosis (i.e. vessel wall thickness and movement): combined with the low costs, rapid development and ease of genetic manipulation, zebrafish are an attractive model for visualisation of inflammatory processes related to early atherogenesis. Yet, it remains to be seen whether the atherosclerotic lesions in zebrafish progress towards more advanced or even rupture-prone atherosclerotic lesions.

Conclusions

Despite the long standing tradition of intravital optical imaging to study leukocyte recruitment, such methods have only started to be employed on a more routine basis by mainstream physiologists and immunologists. This trend in conjunction with the rapid evolution of novel imaging and labelling techniques has emerged in refinement of the classical leukocyte recruitment cascade. Further advances with regard to tissue specific recruitment cues and novel optical methods will pave the way to specific target identification and subsequent development of new therapeutic strategies.

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References

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