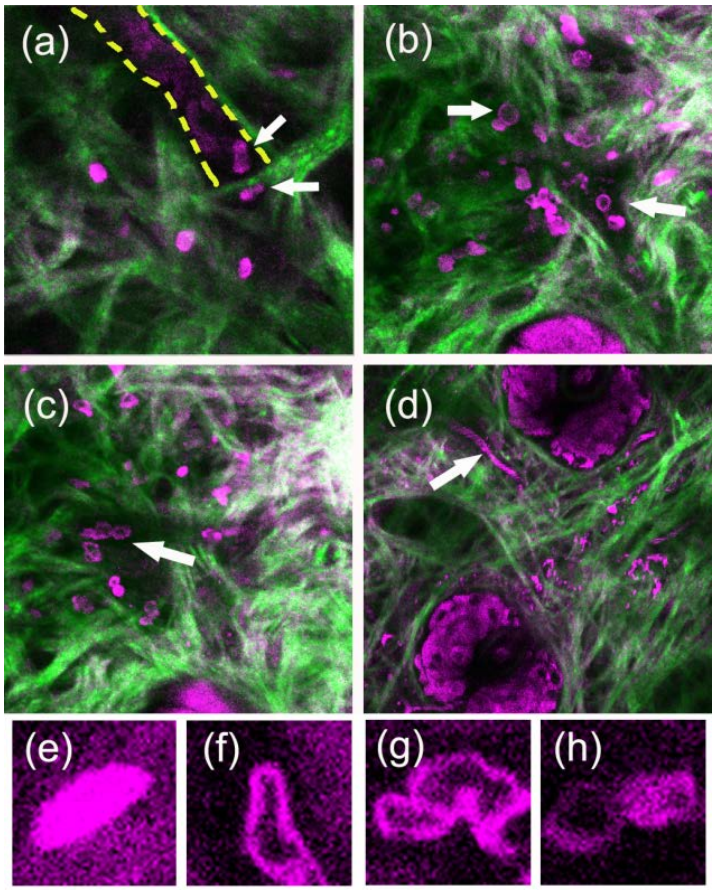

Fig. 4. Combined second harmonic generation (green color) and third harmonic generation (magenta color) time course images of inflammation microenvironments (a) 6 hours, (b),(c) 3 days, and (d) 6 days post-LPS challenge in the same mouse. White arrows indicate (a) deformed neutrophils, (b) hollow-core lymphoid cells, (c) deformed lymphoid cells, and (d) a vessel with circulating red blood cells. Bottom rows shows THG images of elongated (e) neutrophil and (f) lymphoid leukocytes, (g) ruffled lymphoid leukocytes, and (h) cell-cell contact between granulocytes and lymphoid cells. Fields of view are 120 × 120 μm in (a-d) and 12 × 12 μm in (e-h).

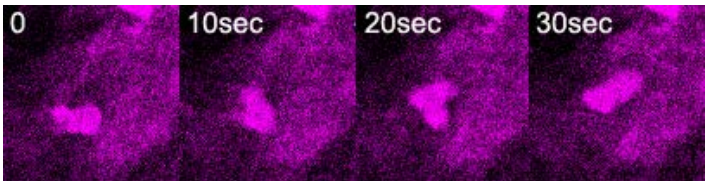

Fig. 5. Amoeboid movement of neutrophils at 3 days post-LPS challenge in Balb/c mice. Field of view: 24 × 24 μm.

To demonstrate how cell/stage statistics can be tracked in this platform, we differentiated cell types in the microenvironment of LPS challenge according to the THG features that we observed *in vitro*. Since monocytes will become macrophages when they enter tissues, we focus on the neutrophil-like (N) and lymphocyte-like (L) cells *in vivo* (Fig. 6). At time points of 10hours, 36hours, and 60hours post LPS challenge, the ICR mouse was analyzed by *in vivo* THG microscopy using the harmonic generation microscopy system. At each time point, we searched for three different places with leukocyte infiltration in mouse ear pinna. We then

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took a stack of 3D sectioning on that site to acquire leukocyte morphology and distributions in three dimensions. Using the THG generated from surrounding collagen, we normalized the average THG intensity of leukocytes over them. By assessing the average intensity levels within cells and the cross sectional intensity profiles, we counted the numbers of neutrophil-like and lymphocyte-like cells. According to the statistical results, the collagen-normalized THG of neutrophil-like cells ranged from 1.44 to 1.8, which somewhat depended on the intensity of THG signals from collagen. The collagen-normalized THG of lymphocyte-like cells (from 0.75 to 1.2) was significantly lower than neutrophil-like cells. The THG contrast between neutrophil-like and lymphocyte-like cells was not as large as the *in vitro* results, which might be due to better refractive index matching with extracellular matrices or the background interference from collagen. From 10hours to 60hours post LPS administration, the density of infiltrated cells at LPS sites increased three fold.

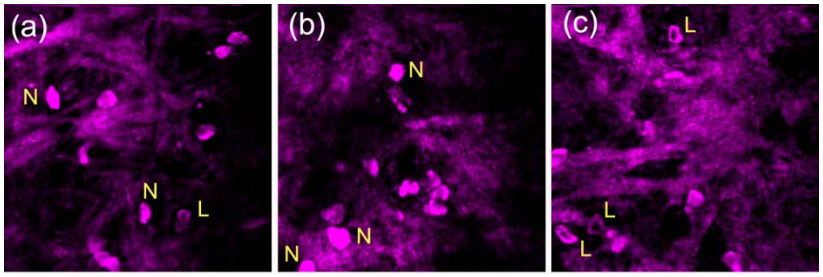

Fig. 6. *In vivo* THG images of leukocyte infiltration in an ICR mouse at (a) 10hours, (b) 36hours, and (c) 60hours post-LPS challenge in ear pinna. Neutrophils (N) and lymphoid cells (L) can be identified in the microenvironment of an immune response. THG signals around leukocytes were generated from collagen, which serves as an *in vivo* reference for the normalization of THG signals from different images.

After counting more than 30 cells, the population percentages of neutrophil-like and lymphocyte-like cells were calculated. According to the statistical results, the population percentage of neutrophil-like leukocytes in the ICR mouse increased from 13% to 20% (Fig. 7) with the progression of an immune response. Lymphocyte-like leukocytes also increased from 5.15 to 12%, though less than neutrophil-like cells.

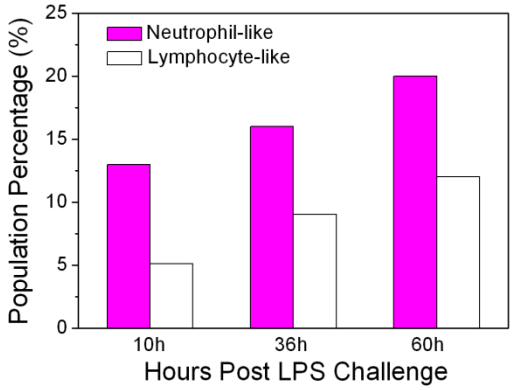

Fig. 7. Population percentages of neutrophil-like (magenta color bar) and lymphocyte-like (white color bar) leukocytes at 10hours, 36hours, and 60hours post-LPS challenge.

There was clearly a higher number of neutrophil-like leukocytes than lymphocyte-like cells, which is in agreement with the fact that neutrophils are the most abundant leukocytes in peripheral blood during first-line innate immune responses. This statistical result demonstrates that our platform of THG microscopy can be used to preliminarily evaluate the immune

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dynamics of different types of leukocytes. However, labeling is still required to identify specific cell identities.

5. Discussion and conclusion

For *in vivo* microscopy, harmonic generation contrasts, such as SHG and THG, are usually considered to be modalities without molecular specificity. However, here we demonstrated that they provide a basic and useful framework through which spatial contexts, such as the distance to vessels, collagen morphologies, and interaction with resident cells can be monitored and measured in microenvironments. Employing harmonic generation contrast together with multiphoton fluorescence microscopy can provide more information for the study of complex immune responses among different types of leukocytes. We also showed that THG microscopy is sensitive to the granularity differences among leukocytes. The contrast originates from the lipid bodies or vesicles inside the granule-rich leukocytes. Through *in vitro* experiments, we found that high granularity neutrophils have extraordinarily strong THG contrast compared to agranular lymphocytes, indicating a potential index to preliminarily classify the lineage of leukocytes. In addition, using *in vivo* THG microscopy, we can determine when and where neutrophil-like and lymphocyte-like leukocytes are recruited in the microenvironments of the LPS challenge. Their sizes, morphologies, trafficking properties, and the cell-cell relationships can all be time-course recorded. These results suggest that label-free THG imaging may provide timely tracing of leukocyte movement without disturbing the normal cellular or physiological status. With further investigation and validation by antibody labeling or cytometry assays, it is possible that such granularity traits of THG can assist in the lineage differentiation of leukocytes and have the potential to advance the study of research topics, such as the immune privilege of stem cells [22], or to capture tumor-immune or tumor-stromal cell interactions in metastatic microenvironments [23].

For clinical diagnosis, these granularity traits can help THG virtual optical biopsy [12–14] identify leukocytes in inflammation sites of skin or oral mucosa. To examine leukocytes in the interior of a hollow organ or cavity of the body, fiber-based THG endoscopy is required. Compared with other techniques, such as μ-OCT or spectrally-encoded microscopy, technical challenges for the implementation of endoscopy include miniaturized high-NA (NA > 0.8) focusing objectives, fibers with low nonlinearity, good THG collection efficiency with the same fiber, miniaturized scanning mechanism, and a compact laser system. In our preliminary studies, we have built a compact Cr:forsterite laser system [24], miniaturized the scanning system by a micro-electro-mechanical system mirror [25,26], used a multimode fiber to reduce pulse broadening [26], and successfully obtained sufficient THG signals through the same fiber [26]. Therefore, we believe THG endoscopy for the examination of leukocytes in the human body can be realized in the near future.

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