A Color-coded Imageable Syngeneic Mouse Model of Stromal-cell Recruitment by Metastatic Lymphoma

TAKURO MATSUMOTO[1], ATSUSHI SUETSUGU[1,2,3], YUHEI SHIBATA[1], NOBUHIKO NAKAMURA[1], HITOMI AOKI[1], TAKAHIRO KUNISADA[1], HISASHI TSURUMI[1], MASAHITO SHIMIZU[1] and ROBERT M. HOFFMAN[2,3]

[1]*Gifu University Graduate School of Medicine, Gifu, Japan;*
[2]*AntiCancer Inc., San Diego, CA, U.S.A.;*
[3]*Department of Surgery, University of California, San Diego, CA, U.S.A.*

Abstract. *Aim: A syngeneic color-coded imageable lymphoma model has been developed to visualize recruitment of host stromal cells by malignant lymphoma during metastasis. The EL4 cell line was previously derived from a lymphoma induced in a C57/BL6 mouse by 9,10-dimethyl-1,2-benzanthracene. Materials and Methods: EL4 lymphoma cells expressing red fluorescent protein (EL4-RFP) were initially established. EL4-RFP cells were subsequently injected into the tail vein of C57/BL6-GFP transgenic mice. Results: EL4-RFP metastasis was observed in the lymph nodes of the upper mediastinum and in the liver 28 days after cell injection. Large EL4-RFP liver metastases in C57/BL6-GFP mice contained GFP-expressing stromal cells derived from the host. In addition, EL4-RFP lymphoma metastasis was formed in peri-gastric lymph nodes, which were also enriched in host GFP-expressing cells. Furthermore, EL4-RFP lymphoma cells were also observed in the peripheral blood and bone marrow of C57/BL6-GFP transgenic mice, where they were associated with GFP-expressing host cells. Conclusion: Lymph node, liver and bone marrow metastases were found approximately 4 weeks after transplantation and all RFP-expressing metastases were highly enriched in GFP-expressing host stromal cells. This model of malignant lymphoma can be used to study early tumor development, metastasis, and the role of the stroma, as well as for discovery and evaluation of novel therapeutics for this treatment-resistant disease.*

This article is dedicated to the memory of A. R. Moossa, MD.

Correspondence to: Robert M. Hoffman, Ph.D., AntiCancer Inc., 7917 Ostrow Street, San Diego, CA 92111, U.S.A. Tel: +1 8586542555, Fax: +1 8582684175, e-mail: all@anticancer.com and Atsushi Suetsugu, MD, Ph.D., Department of Gastroenterology, Gifu University Graduate School of Medicine, Gifu, Japan, 1-1 Yanagido, Gifu 501-1194, Japan. Tel: +81 582306308, Fax: +81 582306310, e-mail: asue@gifu-u.ac.jp

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With the use of multiple colored proteins, we have developed imaging of the tumor microenvironment (TME) by color-coding cancer and stromal cells (1-21). The TME is necessary for tumor growth and progression to occur (5). Color-coded imaging technology of the TME used green fluorescent protein (GFP) (2), red fluorescent protein (RFP) (7), or cyan fluorescent protein (8) transgenic nude mice as hosts into which we transplanted cancer cells expressing a fluorescent protein not expressed by the host (1-21).

Tumors contain fibroblasts, lymphocytes, dendritic cells, macrophages and other myeloid cells in their microenvironment (22). Cancer-associated fibroblasts (CAFs) stimulate cancer cell growth, inflammation, angiogenesis, and invasion (22-26). As a tumor grows, it recruits CAFs and other host cells (27, 28). We reported the importance of CAFs in metastasis to the liver (9, 12). Non-colored HCT-116 human colon cancer cells were injected into the spleen of GFP-expressing nude mice which led to the formation of experimental liver metastases. GFP-expressing host cells were recruited by the metastatic tumors as visualized by fluorescence imaging. A desmin-positive area increased around and within the liver metastasis over time, suggesting CAFs were recruited by the liver metastasis, and appeared to have a role in its progression (9).

How the TME is formed in real time during progression and metastasis of malignant lymphoma is poorly understood. The present study utilized color-coded fluorescent protein-based imaging to visualize the recruitment over time of host cells by lymph-node and liver metastases of a malignant lymphoma in a syngeneic mouse model.

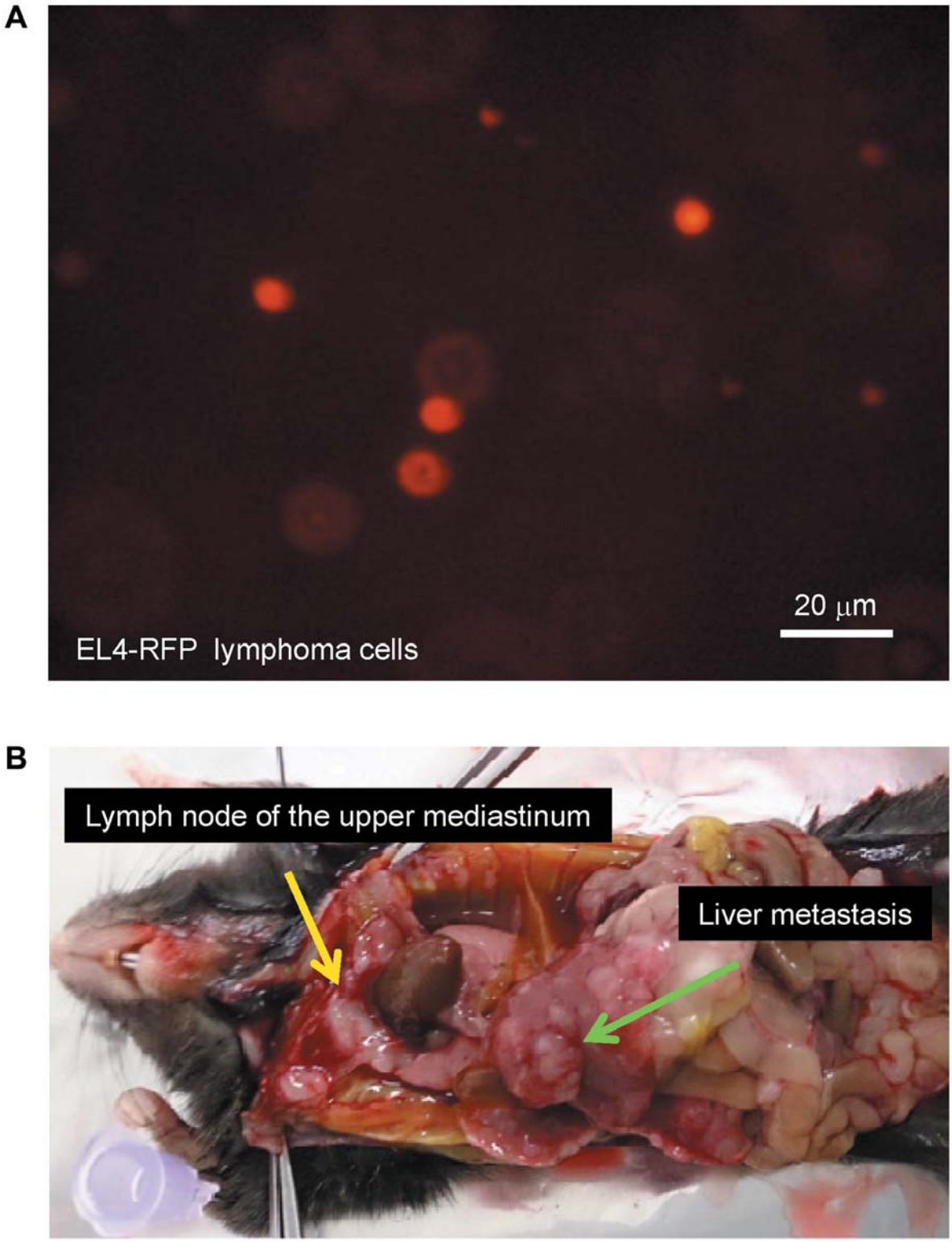

Figure 1. *EL4, a mouse lymphoma cell line was established from a lymphoma induced in a C57/BL mouse by 9,10-dimethyl-1,2-benzanthracene (29). A: EL4-red fluorescent protein (RFP) mouse lymphoma cancer cells were established. Visualization of EL4-RFP mouse lymphoma cancer cells in vitro. EL4-RFP mouse lymphoma cancer cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum. Images were captured with an Olympus IX71 microscope. B: Brightfield image of metastasis in a green fluorescent protein (GFP)-expressing mouse. Yellow arrow indicates lymph node metastasis of the upper mediastinum. Green arrow indicates liver metastasis.*

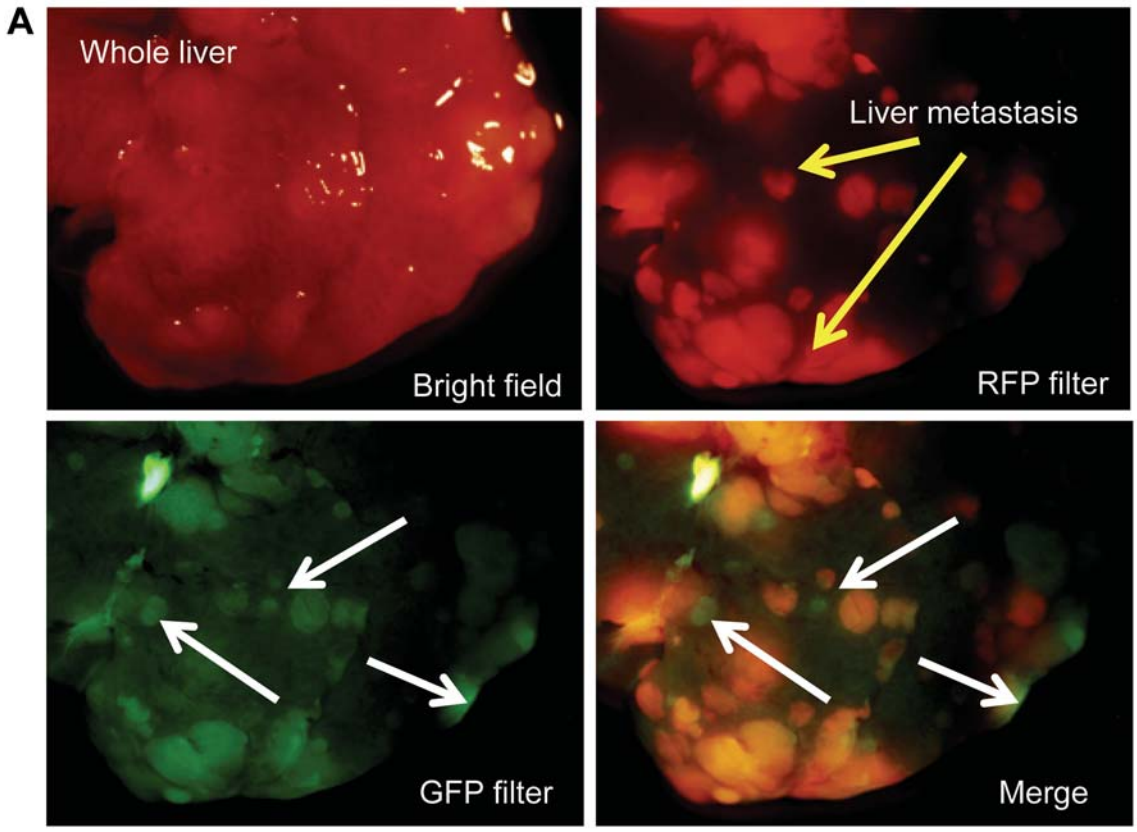

Figure 2. *Continued*

Materials and Methods

Cell line and culture conditions. EL4, a mouse lymphoma cell line was established from a lymphoma induced in a C57/BL6 mouse by 9,10-dimethyl-1,2-benzanthracene (29). The cells were maintained in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin and streptomycin (Gibco-BRL, Grand Island, NY). The cells were cultured at 37°C in a 5% CO_2 incubator.

RFP fluorescent protein transduction of lymphoma cells. Mouse EL4 lymphoma cells were labeled with RFP as previously reported using retroviruses expressing RFP (Figure 1A) (4, 30, 31).

GFP transgenic mice. Transgenic C57/BL6-GFP mice (32) were obtained from the Research Institute for Microbial Diseases (Osaka University, Osaka, Japan). The C57/BL6-GFP mice expressed *Aequorea victoria* GFP under the control of the chicken β-actin promoter and cytomegalovirus enhancer.

Color-coded lymphoma-host cell model. Six-week-old GFP immunocompetent C57/BL6-GFP transgenic mice were used as the host for EL4-RFP lymphoma cells. EL4-RFP lymphoma cells were first harvested by trypsinization and washed three times with cold serum-free medium and then resuspended with serum-free RPMI-1640 medium. EL4-RFP lymphoma cells were injected into the tail vein of C57/BL6-GFP transgenic mice.

Tumor imaging. An IX70 microscope, SZX7 microscope, and FV1000 confocal microscope, all from Olympus Corp. (Tokyo, Japan), were used in this study.

Study approval. All experiments were conducted in accordance with the institutional guidelines of Gifu University and were approved by the Animal Research Committee and the Committee on Living Modified Organisms of Gifu University (approval number 26-37).

Results and Discussion

Imaging of recruitment of host cells by malignant lymphoma cancer metastasis. Red fluorescent protein (RFP)-expressing EL4 mouse lymphoma cells were established (Figure 1A). The EL4-RFP cells were injected into the tail vein of GFP-expressing nude mice. EL4-RFP metastasis was observed in the lymph nodes of the upper mediastinum and in the liver 28 days after cell injection (Figure 1B). High-magnification fluorescence microscopy demonstrated extensive GFP host cells in the metastasis.

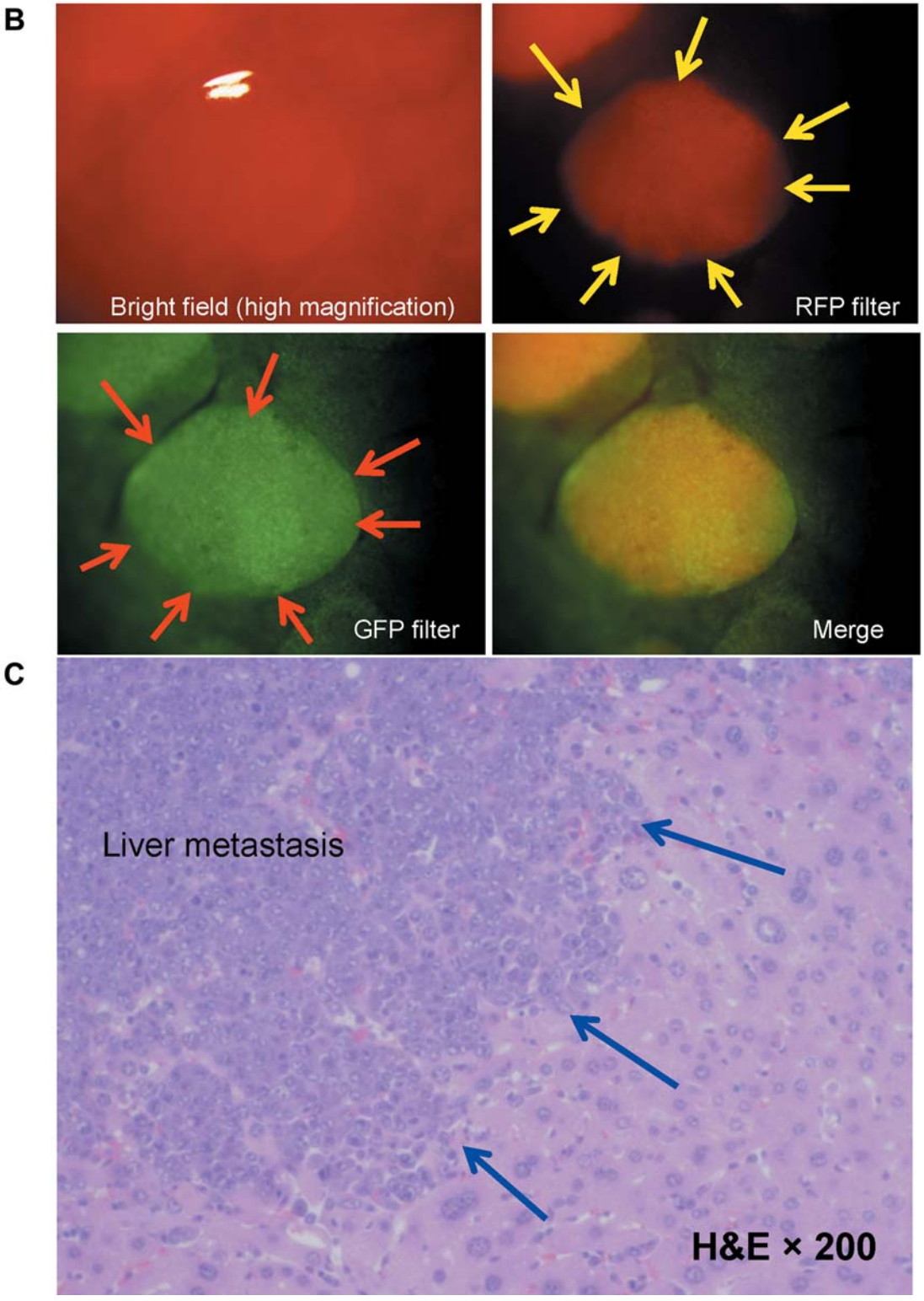

Figure 2. *(Continued from previous page) A: Bright-field and fluorescence images of a liver metastasis from EL4- red fluorescent protein (RFP) mouse lymphoma 28 days after tail-vein injection of the cells. Yellow arrows indicate liver metastasis. White arrows indicate green fluorescent protein (GFP)-expressing host cells. B: High-magnification imaging of liver metastasis. Yellow arrows indicate EL4-RFP tumor. Red arrows indicate GFP-expressing host cells. Images were captured with an Olympus SZX7 microscope. C: Liver section from a GFP-expressing mouse was stained with hematoxylin and eosin. Blue arrows indicate EL4 mouse lymphoma cells.*

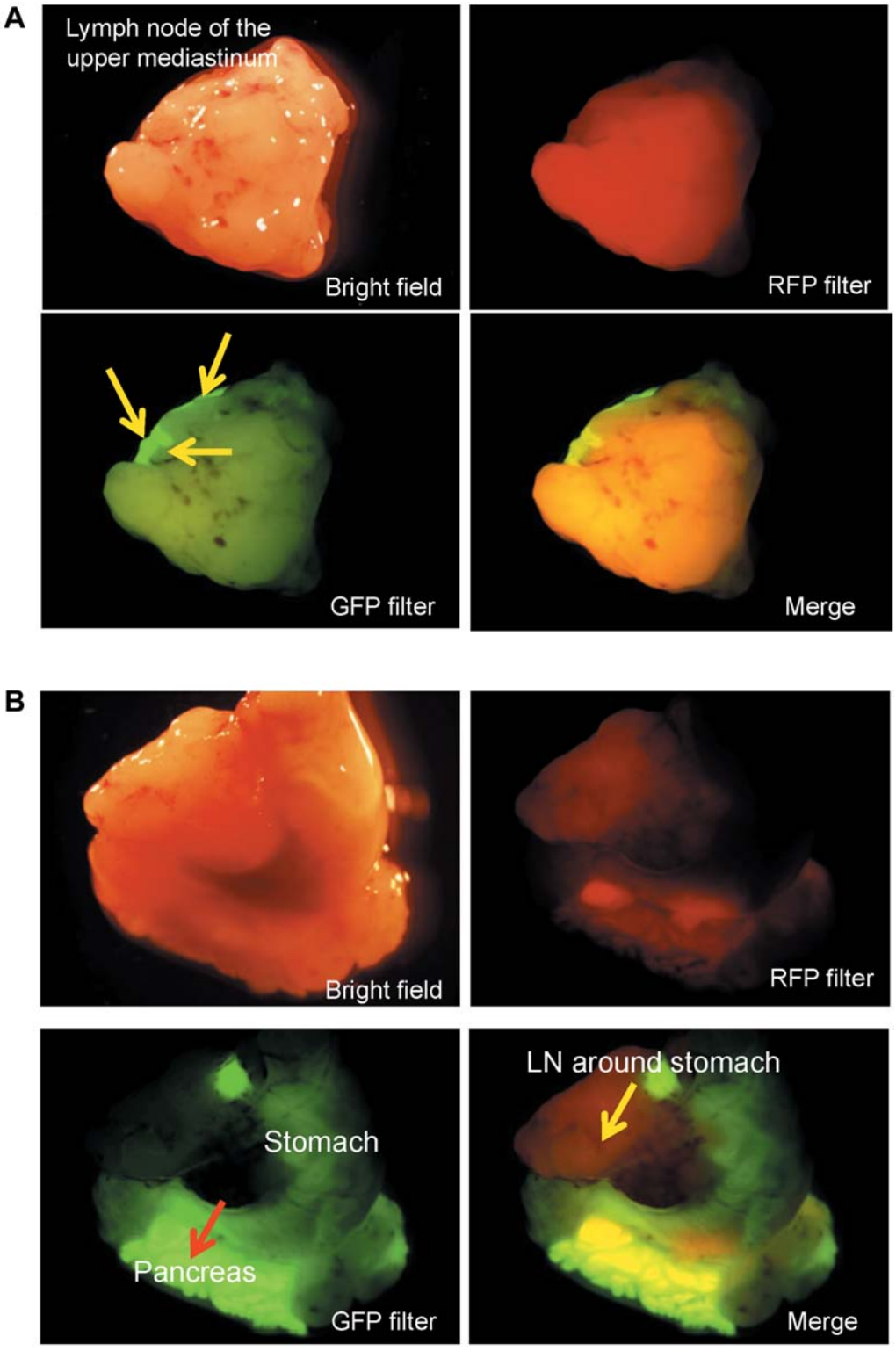

Figure 3. *(Continued from previous page) A: Bright-field and fluorescence images of metastatic lymph nodes of the upper mediastnum 28 days after tail-vein injection of EL4- red fluorescent protein (RFP) mouse lymphoma cells. Yellow arrows indicate green fluorescent protein (GFP)-expressing host cells. Images were captured with an Olympus SZX7 microscope. B: Bright-field and fluorescence images of metastatic lymph nodes around the stomach 28 days after tail-vein injection of EL4-RFP mouse lymphoma cells. Yellow arrows indicate lymph node metastasis around the stomach. Red arrows indicate bright GFP in the pancreas of the GFP-expressing mouse.*

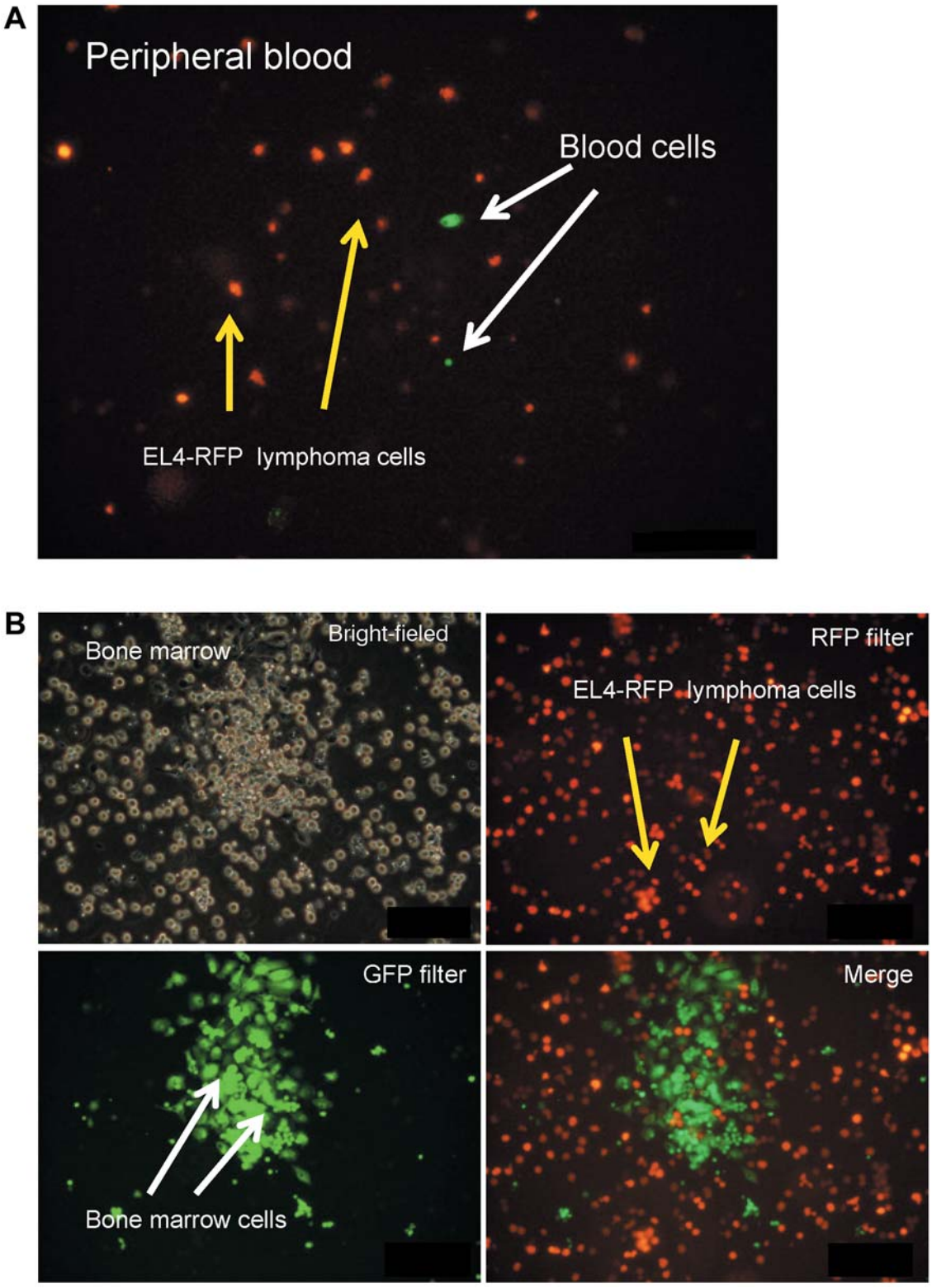

Figure 4. *A: Peripheral blood cells were collected from green fluorescent protein (GFP) transgenic mice with the EL4- red fluorescent protein (RFP) lymphoma. The EL4-RFP cells circulated in the peripheral blood. White arrows indicate GFP-expressing blood cells. Yellow arrows indicate EL4-RFP lymphoma cells. B: Bone marrow cells were removed from C57/BL6-GFP transgenic mice. EL4-RFP lymphoma cells (yellow arrows) were observed in the bone marrow, along with GFP-expressing bone marrow (white arrows) of the C57/BL6-GFP transgenic mice.*

GFP fluorescence of recruited GFP-expressing hosts cells in the liver metastasis was observed (Figure 2 A and B). There was a very large increase of GFP expression in the metastasis compared to the normal part of the liver in GFP-expressing mice due to GFP host cell recruitment by the metastasis. Host GFP-expressing cells were imaged extensively accumulating in the liver metastasis. Large EL4-RFP liver metastases in C57/BL6-GFP mice had GFP-expressing stromal cells derived from the host animal around the edge of EL4-RFP tumors.

EL4-RFP lymphoma metastasis was formed in mediastinal lymph nodes (Figure 3A) and a peri-gastric lymph node (Figure 3B). Similarly, GFP host cells were visualized around the mediastinal and peri-gastric lymph-node metastases.

EL4-RFP lymphoma cells circulated in the peripheral blood along with GFP-expressing blood cells (Figure 4A). Furthermore, EL4-RFP lymphoma cells were observed in bone marrow of C57/BL6-GFP transgenic mice near GFP-expressing bone marrow cells (Figure 4B).

Liver and lymph node sections from GFP mice were stained with hematoxylin and eosin and both normal and malignant lymphoma cells could be seen (Figure 2C and 3B).

We demonstrate a color-coded imaging model in which the development of the TME during lymphatic metastasis can be visualized in a syngeneic model of metastatic lymphoma. In GFP-expressing transgenic mice, only non-parenchymal cells of the liver have GFP fluorescence, which makes it a very useful model for imaging stromal recruitment by liver metastases. This model of malignant lymphoma can be used to study early tumor development, metastasis and the role of stroma, and for the discovery and evaluation of novel therapeutics for targeting both the lymphoma and its host stromal cells.

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