Journal of Innate Immunity

Research Article

J Innate Immun 2023;15:442–467
DOI: 10.1159/000529096

Received: August 19, 2022
Accepted: January 9, 2023
Published online: March 30, 2023

Single Cell Analysis of the Fate of Injected Oncogenic RasV12 Cells in Adult Wild Type *Drosophila*

Di Chen[a] Xiao Lan[a] Xiaoming Huang[a] Jieqing Huang[a] Xiaojing Zhou[a] Zhichao Miao[b, c] Yuting Ma[d, e] Akira Goto[f] Shanming Ji[f] Jules A. Hoffmann[a, f, g]

[a]Sino-French Hoffmann Institute, School of Basic Medical Science, State Key Laboratory of Respiratory Disease, Guangzhou Medical University, Guangzhou, China; [b]Guangzhou Laboratory, Guangzhou International Bio Island, Guangzhou, China; [c]Translational Research Institute of Brain and Brain-Like Intelligence and Department of Anesthesiology, Shanghai Fourth People's Hospital Affiliated to Tongji University School of Medicine, Shanghai, China; [d]Institute of Systems Medicine, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China; [e]Suzhou Institute of Systems Medicine, Suzhou, China; [f]Université de Strasbourg and CNRS, Insect Models of Innate Immunity (M3I; UPR9022), Strasbourg, France; [g]University of Strasbourg Institute for Advanced Study, Strasbourg, France

Keywords
Oncogenic cells · Destiny after injection · *Drosophila*

Abstract
We have injected dish-cultured oncogenic RasV12 cells into adult male flies and analyzed by single cell transcriptomics their destiny within the host after 11 days. We identified in the preinjection samples and in the 11-day postinjection samples in all 16 clusters of cells, of which 5 disappeared during the experiment in the host. The other cell clusters expanded and expressed genes involved in the regulation of cell cycle, metabolism, and development. In addition, three clusters expressed genes related to inflammation and defense. Predominant among these were genes coding for phagocytosis and/or characteristic for plasmatocytes (the fly equivalent of macrophages). A pilot experiment indicated that the injection into flies of oncogenic cells, in which two of most strongly expressed genes had been previously silenced by RNA interference, into flies resulted in a dramatic reduction of their proliferation in the host flies as compared to controls. As we have shown earlier, the proliferation of the injected oncogenic cells in the adult flies is a hallmark of the disease and induces a wave of transcriptions in the experimental flies. We hypothesize that this results from a bitter dialogue between the injected cells and the host, while the experiments presented here should contribute to deciphering this dialogue.

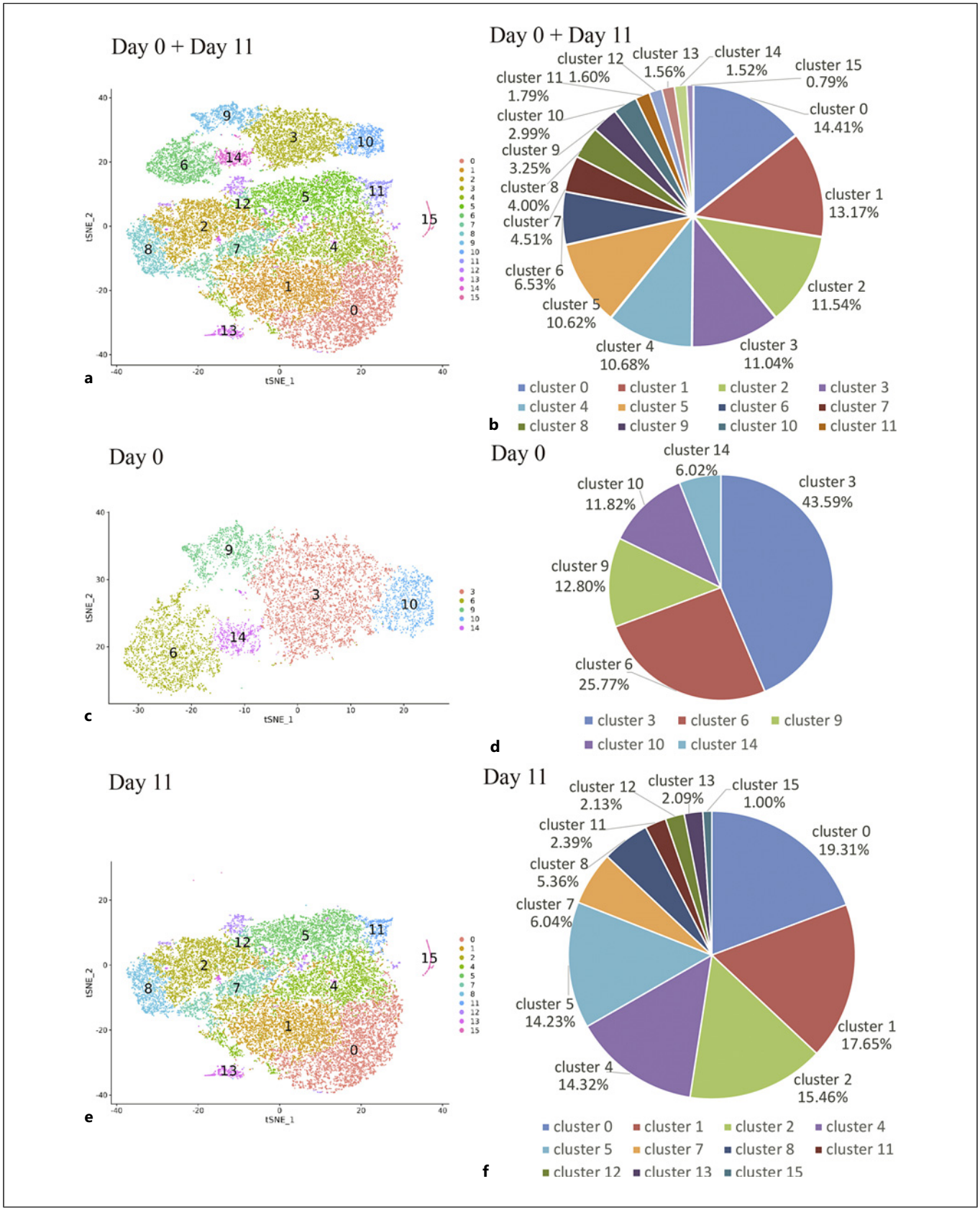
Introduction

Flies have been the subject in recent decades of intense investigations of their defense reactions against various types of microbes [1–6]. These studies have

Di Chen and Xiao Lan are co-first authors.

karger@karger.com
www.karger.com/jin

Correspondence to:
Di Chen, chenseptember@outlook.com
Jules A. Hoffmann, j.hoffmann@unistra.fr



1

(For legend see next page.)

generated a global picture regarding both the receptors recognizing the invading germs and the signaling cascades initiated by the various categories of microbes. Further, many of the molecules which are produced in response to these infections have been identified and their mode of action analyzed [7, 8]. Of interest to the community of immunologist in particular was the realization that innate immunity in insects shares significant similarities with the innate immune arm of the host defenses of mammals [9]. Flies are not only challenged by microbes but are also confronted by cancer cells, as most eukaryotes. As regards *Drosophila*, the developments of cancers have been reported more than a century ago [10–12] and have been the subject of many in-depth analyses, mainly focusing on their genetic and molecular origins [13–17]. We have recently addressed the question whether the defense reactions against this type of endogenous genetic insult would induce responses similar to those observed during infections by external germs. There are obviously several ways to approach this question and we have opted for the injection of oncogenic GFP-labeled cells from a *Drosophila* immortalized cell line [18, 19] into adult male flies. Our prime objective was to understand the host reactions to the injected cells and the destiny of these injected cells in this system. We anticipated that on the long run this approach would yield a clear picture of the potential dialogue between the host and the injected cells over an extended period, up to the demise of one or both participants.

The injected RasV12 oncogenic cell (OC) line which we used here and in our previous study [20] is a tumor cell line of embryonic origin, constructed by A. Simcox and colleagues in 2008 through introducing an oncogenic form of Ras (RasV12, carrying the Val/Gly mutation) into *Drosophila* embryos under a constitutive *Actin 5C* promoter which also controls expression of the gene encoding the reporter GFP. The transgenic cells were isolated by these authors and continuously passaged over 20 generations for immortalization [18]. In 2015, Dequéant and colleagues, using the same model, performed a time-series transcriptional synexpression analysis on different passages from several independent immortalization processes. They found that the bulk of immortalized cells was related to adult muscle precursors, i.e., a stem cell-like population contributing to adult muscles and sharing properties with vertebrate satellite cells [19]. Neither group had the objective of following the destiny of these cells after injection into flies, which is precisely our objective in the present study.

A previous report from our group, based on the same immortalized cell line which we use here, and performed in the same experimental setting, had pointed to major differences between antimicrobial defenses and reactions against homologous OCs [20]. Here, we extend these data by using single-cell transcriptomics of the injected cells at the time of injection into the flies and after allowing for proliferation for 11 days in the host.

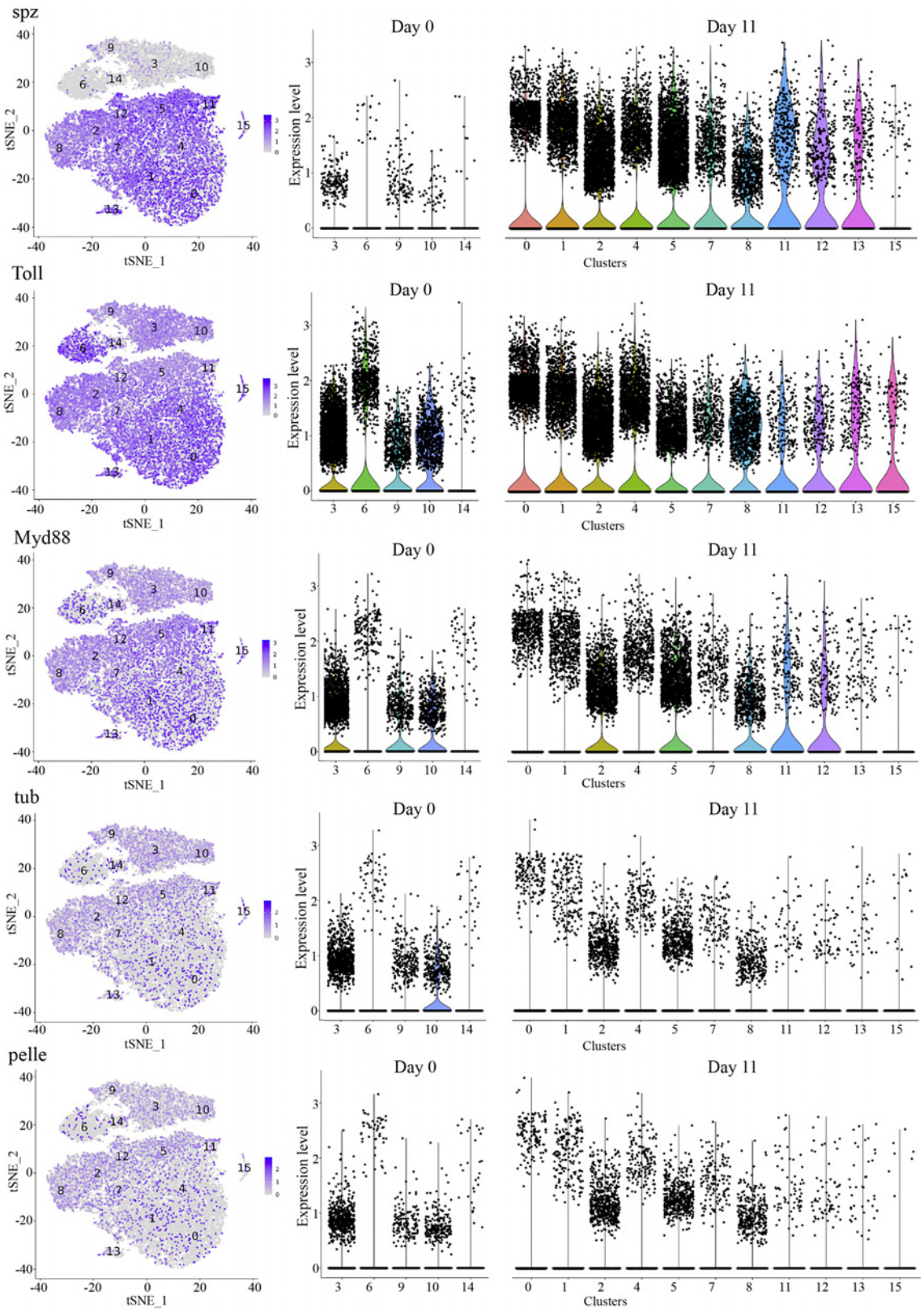
In our previous study based on bulk transcriptomics of experimental flies (i.e., flies having received an injection of RasV12 OCs), we observed on day 3 postinjection a significant transcriptomic response of the flies to the injection of the OCs. This response was followed a few days' dormancy by exponential increase of the OCs. We wondered whether this phenomenon may relate to the heterogeneity of the injected OCs which we used. We therefore decided to follow the destiny of the injected cells by single-cell transcriptomics. Here, we report that we could differentiate between 16 clusters among the cells used in these experiments. From the time of injection into the flies up to day 11, several of these clusters disappeared as such whereas others expressing different transcriptomic profiles became apparent. As could be expected, most cells in these various clusters express genes reported to be involved in cell cycle regulation, in development, in metabolism, energy production, etc. We provide here a detailed description of the various clusters and of their relative importance during the period of the experiment. Importantly, in the context of our present interest, three clusters express massively genes involved in various processes of inflammation. In contrast, we observed only very few cells in these clusters which expressed genes encoding antimicrobial peptides (AMPs).

Materials and Methods

Fly Strain

W^{1118} flies were reared on standard cornmeal-yeast-agar medium at 25°C under 60% humidity.

Fig. 1. 16 clusters and their proportions identified for the day 0 and day 11 RasV12 samples by single cell deep sequencing. **a** t-SNE projection of the 16 clusters identified from the pool of cells from day 0 to day 11 samples with a resolution of 0.8. Day 0 sample represents RasV12 oncogenic cells collected from dish-culture before injection. Day 11 sample represents RasV12 oncogenic cells isolated from 100 adult male *Drosophila* 11 days after injection. **b** Proportions of 16 clusters of cells from day 0 to day 11 samples. **c** t-SNE projection of cells from day 0 sample. **d** Proportions of clusters from day 0 sample. **e** t-SNE projection of cells from day 11 sample. **f** Proportions of clusters from day 11 sample.



2

(Figure continued on next page.)

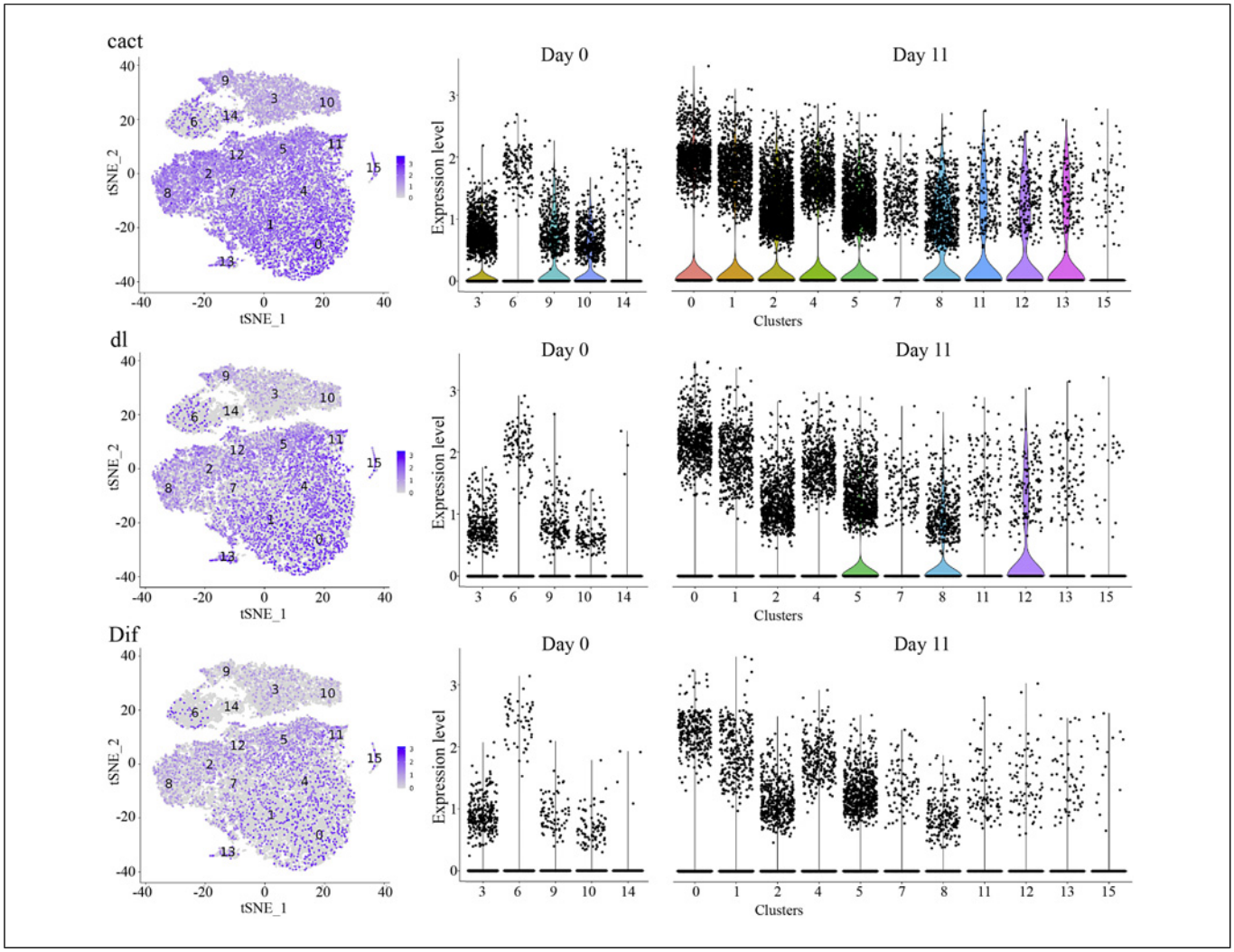


Fig. 2. Feature plots and violin plots for genes in Toll pathway for each cluster of day 0 and day 11 sample. Feature plot (picture on the right): the indicated gene expression on each cell was labeled with blue color dot. The relative expression level of each gene was indicated by the intensity of color blue. Cells will be labeled as gray color when their expression levels of indicated gene are non-detectable. Violin plots (picture on the left): The *Y* axis marked the expression level of the indicated genes. The *X* axis represents each cluster of day 0 sample and day 11 sample. The indicated gene expression on each cell was labeled with one black dot.

Cell Culture

The RasV12-GFP cells were grown in Schneider's medium (Thermo) supplemented with 10% FBS (Gibco), 1% Glutamax (Invitrogen), and 1% penicillin/streptomycin (Invitrogen) in 25°C without CO_2.

Injection of RasV12 Cells into Flies

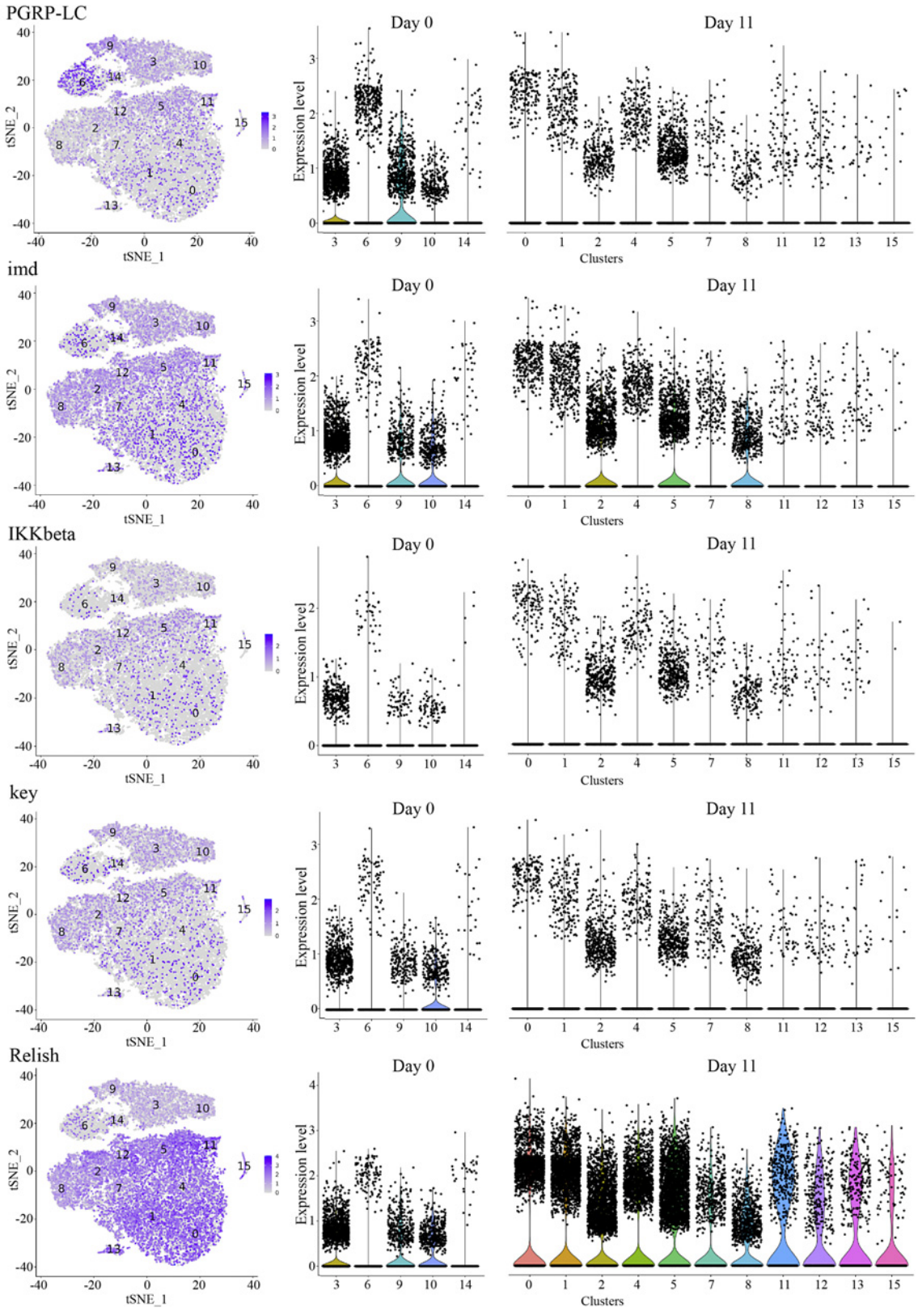
500 cells/10 nL/fly were injected into the thorax of CO_2-anesthetized adult male flies using a nanoliter injector (Nanoject III, Drummond). The injected flies were raised at 25°C up to 11 days after injection.

Dissociation for Single Cell Deep Sequencing

Dish-cultured cells were harvested as day 0 sample. For day 11 sample, flies receiving RasV12 cells were reared at 25°C for 11 days and were dissected in cold PBS in a 6 cm dish. The fragmented tissues were then incubated in PBS with a mixture of 1 mL DISPASE (SIGMA D4639) and preheated Collagenase II (SIGMA C6885) and Collagenase IV (SIGMA C5138) at a final concentration of 2 mg/mL. The mixture was placed on a shaker for 20–30 min (150 rpm/min) at room temperature. After dissociation, the mixture was filtered (40 μm strainer) on ice and washed at 400 g for 4 min twice. The suspended cells were sorted by a BD FACS Arria II cell sorter according to their GFP fluorescence. Dead cells were excluded by DAPI. GFP^+DAPI^- cells were collected for day 11 sample.

Chromium 10× Genomics Library and Sequencing

Single-cell suspensions were loaded on a 10× Chromium to capture single cell according to the manufacturer's instructions of



3

(For legend see next page.)

10× Genomics Chromium Single-Cell 3' kit (V3). The following cDNA amplification and library construction steps were performed according to the standard protocol. Libraries were sequenced on an Illumina NovaSeq 6000 sequencing system (paired-end multiplexing run, 150 bp) by LC-Bio Technology Co.Ltd. (HangZhou, China).

Bioinformatics Analysis

Sequencing results were demultiplexed and converted to FASTQ format using Illumina bcl2fastq software (version2.20). Sample demultiplexing, barcode processing, and single-cell 3′gene counting were done by using the Cell Ranger pipeline (https://support.10xgenomics.com/single-cell-gene-expression/software). scRNA-seq data were aligned to Index of/genomes/*Drosophila*_melanogaster (ftp.flybase.net/genomes/*Drosophila*_melanogaster/). A total of 14,879 single cells captured from day 0 sample and 32,726 single cells captured from day 11 sample were processed using 10× Genomics Chromium Single Cell 3′ Solution. The Cell Ranger output was loaded into Seurat (version 3.1.1) to be used for dimensional reduction, clustering, and analysis of scRNA-seq data. To visualize the data, we further reduced the dimensionality of 8,223 cells from day 0 sample and 24,190 cells from day 11 sample using Seurat and t-SNE to project the cells into 2D space. The steps include: 1. use of the LogNormalize method of the "Normalization" function of the Seurat software to calculate the expression values of genes; 2. Principal component analysis was performed using the normalized expression value. Within all the PCs, the top 10 PCs were used to do clustering and t-SNE analysis; 3. To find clusters, we selected weighted Shared Nearest Neighbor graph-based clustering method. Marker genes for each cluster were identified with the Wilcoxon rank-sum test (default parameters is "bimod": Likelihood-ratio test) with default parameters via the FindAllMarkers function in Seurat. This selects marker genes which are expressed in more than 10% of the cells in a cluster and average log (Fold Change) of greater than 0.26.

siRNA Transfection

4×10^5 RasV12 cells were plated in 24-well plate and incubated overnight in 450 μL Schneider's medium. 2.5 μL of 20 μM siRNA stock solution (v3) was diluted with 30 μL of 1X riboFECT™ *CP* Buffer (v2) and mixed gently, and after addition 3 μL of riboFECT™ *CP* Reagent (v4), the mixture was incubated for 10–15 min at room temperature for preparing the transfection complex. We added the riboFECT™ *CP* transfection complex to the cells and mixed gently. Cells were kept at 25°C without CO_2 for 8 h. After that, 50 μL FBS was added into each well and cells were kept at 25°C for 64 h. The sequence of siRNA targeting *Relish* was: GCC TGGTCTTTCAGATGAA, the sequence of siRNA targeting *eater* was: GGATACAAGCTGAATCCAA, the non-targeting siRNA sequence was: GGTTCACCGCTACATTTAA. Reagents and siRNAs were ordered from Ribobio company.

RT-qPCR

Total RNAs from whole fly or RasV12-GFP cells were isolated using RNAex pro Reagent (AG). Briefly, 1 μg of total RNA was reverse transcribed using Evo M-MLV RT Kit with gDNA Clean (AG). 100 ng of cDNA were used for quantitative real time PCR (RT-qPCR) using SYBR Green Premix (AG) on a CFX384 Touch Real-Time PCR platform (Bio-Rad). Normalization was performed with the housekeeping gene *ribosomal protein 49* (*Rp49*). The qPCR data were analyzed by ΔΔCT method. The sequences of primers are shown in online supplementary Table 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000529096).

Statistical Analysis

Unpaired two-tailed Student's *t* test was used for statistical analysis of data with GraphPad Prism (GraphPad Software). Error bars give standard deviations. Where indicated, a two-way analysis of variance (ANOVA), followed by pairwise multiple comparisons, was done. *p* values lower than 0.05 were considered statistically significant.

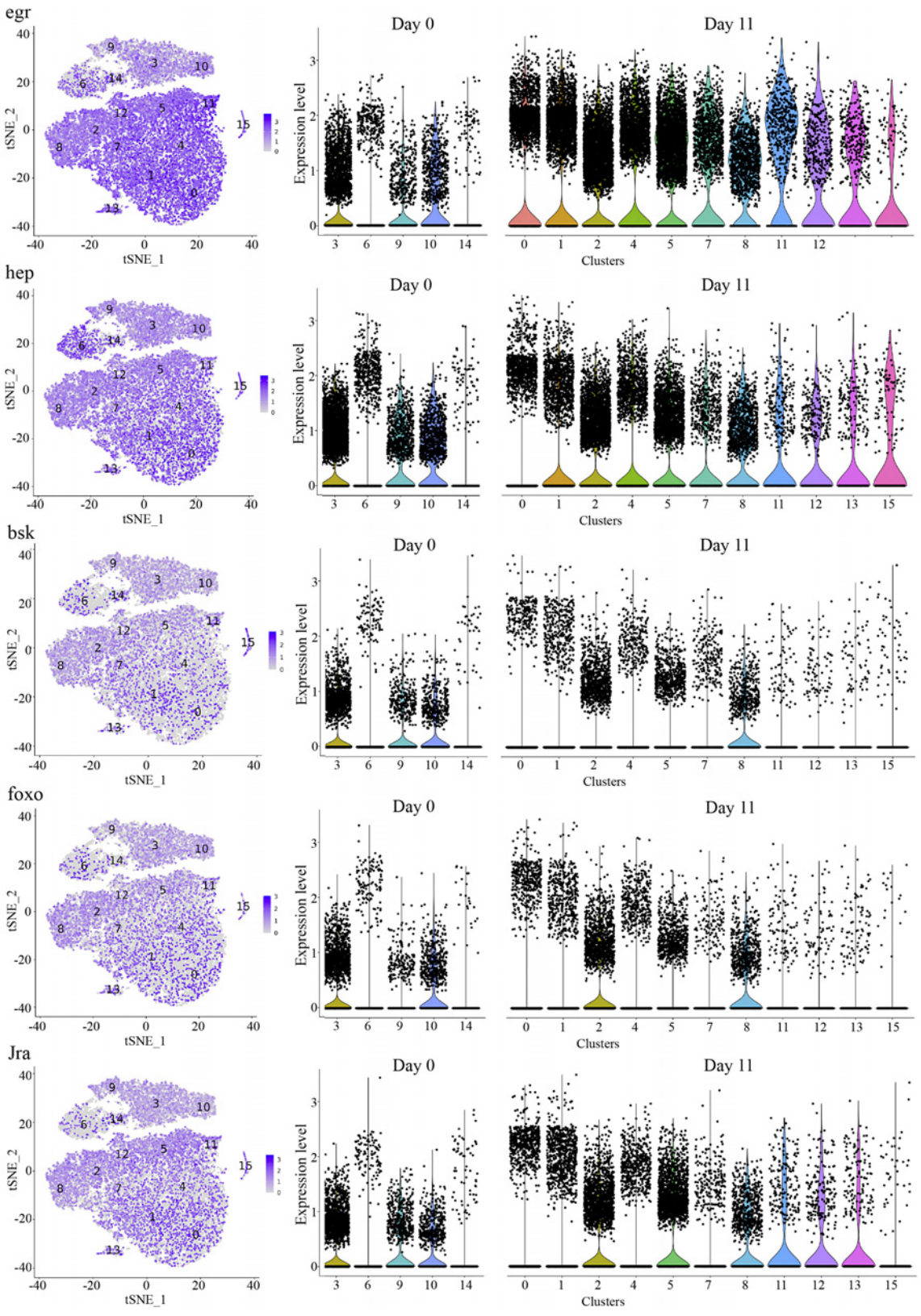
Gene Ontology Enrichment Analyses

Marker genes of each cluster were mapped to gene ontology (GO) terms in the GO database (http://www.geneontology.org/).

Results

For the sake of clarity, we present our results in four subsections. We will first describe the isolation of the combined 16 clusters from day 0 sample (OCs before injection into flies) and from day 11 sample (the clusters isolated from flies 11 days after the injection of the cells). We will next provide an analysis of each cluster based on GO. The third section will be devoted to an in-depth analysis of three day-11 clusters which are marked by a predominance of genes encoding proteins frequently associated with inflammation and/or defense reactions in flies. Finally, we will present in the last section a pilot experiment to validate some of the analyses of these identification/clustering data in the context of our present interests.

Fig. 3. Feature plots and violin plots for genes in IMD pathway for each cluster of day 0 and day 11 sample. Feature plot (picture on the right): The indicated gene expression on each cell was labeled with blue color dot. The relative expression level of each gene was indicated by the intensity of color blue. Cells will be labeled as gray color when their expression levels of indicated gene are non-detectable. Violin plots (picture on the left): The *Y* axis marked the expression level of the indicated genes. The *X* axis represents each cluster of day 0 sample and day 11 sample. The indicated gene expression on each cell was labeled with one black dot.



4

(Figure continued on next page.)

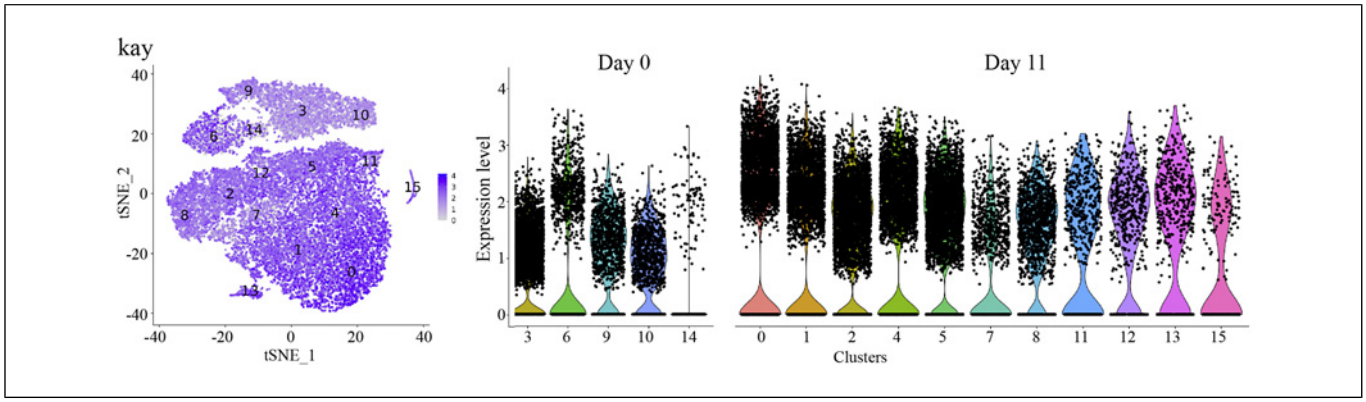


Fig. 4. Feature plots and violin plots for genes in JNK pathway for each cluster of day 0 and day 11 sample. Feature plot (picture on the right): The indicated gene expression on each cell was labeled with blue color dot. The relative expression level of each gene was indicated by the intensity of color blue. Cells will be labeled as gray color when their expression levels of indicated gene are non-detectable. Violin plots (picture on the left): The *Y* axis marked the expression level of the indicated genes. The *X* axis represents each cluster of day 0 sample and day 11 sample. The indicated gene expression on each cell was labeled with one black dot.

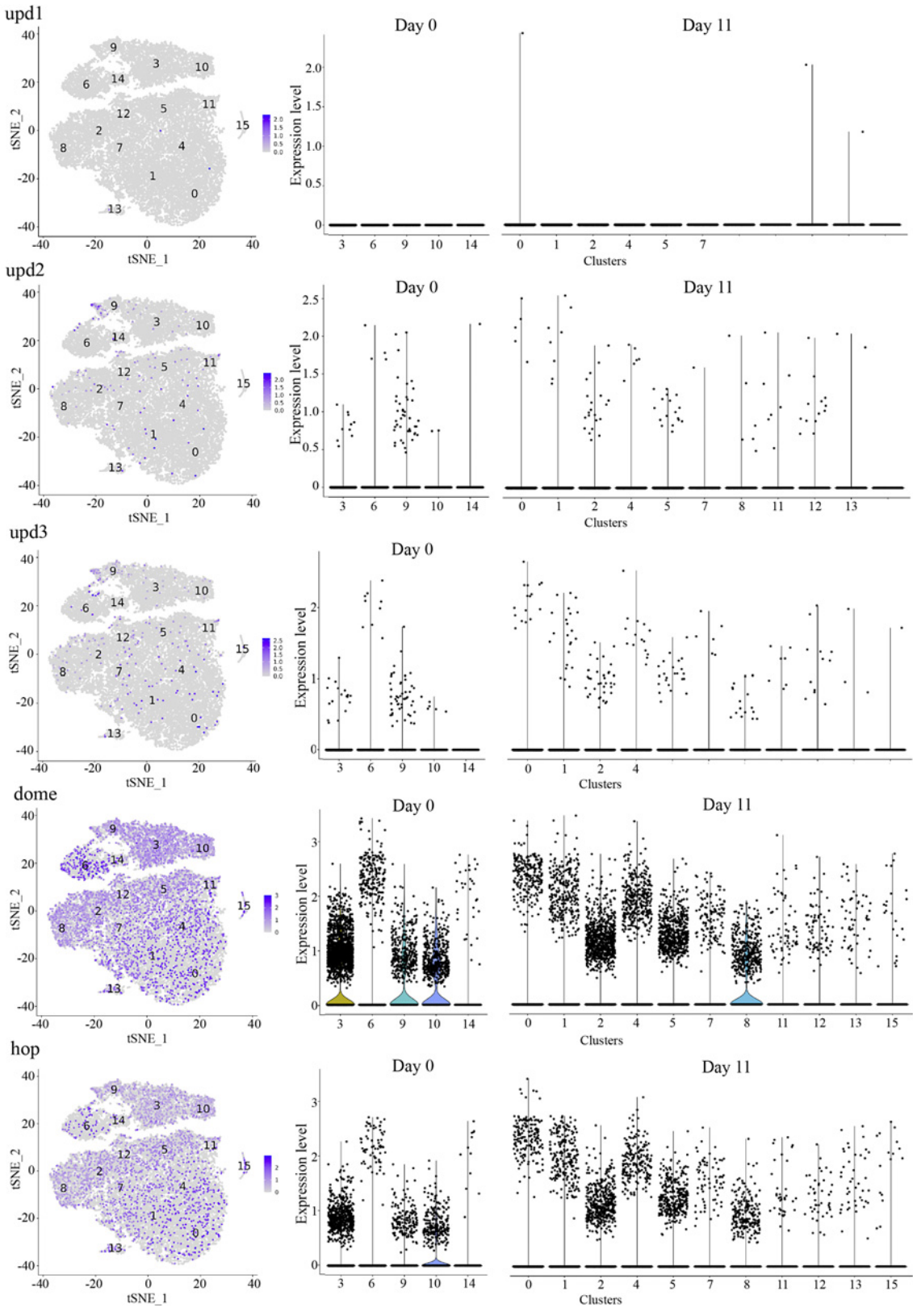
Identification of 16 Clusters of Cells by Single Cell Sequencing

We first investigated by single cell sequencing the transcriptomic profiles of two relevant samples: (1) dish-cultured GFP-labeled RasV12 OCs (hereafter referred to as day 0 sample) collected at the time of their injection into the flies, and (2) RasV12 cells isolated from adult male *Drosophila* 11 days after their injection into the flies (referred to as day 11 sample). As described in detail in our previous report, the injected OCs, after a lag period of 3–5 days, proliferate within the fly body up to day 11 when half of the flies in the experimental group have succumbed as a direct or indirect (or both) result of the injection of the OCs [20]. We collected the cells and the transcriptomic profiles of 8,223 cells served to prepare the library of the day 0 sample, and the profiles of 24,190 cells were used for the library of the day 11 sample. The libraries from both samples were merged and OCs presenting similar expression profiles were clustered using the Seurat toolkit [21, 22]. We identified in total 16 distinct clusters (Fig. 1a, b, clusters 0–15). Of these, five major clusters (3, 6, 9, 10, 14), accounted for more than 99% of the day 0 sample cells (Fig. 1c, d), and six out of eleven clusters (0, 1, 2, 4, 5, 7, and 8) of day 11 represented a total of 92% of the corresponding cells (Fig. 1e, f). Importantly, the t-distributed stochastic neighbor embedding projection (t-SNE) values (Fig. 1a, c, e) indicate that the expression profiles of the absolute majority of cells of the day 11 sample are different from those of the day 0 sample, pointing to a clear evolution of the injected OCs in the host during the 11 days of the experiment.

The comparison of the expression profiles between day 0 and day 11 samples indicates two essential patterns of changes during this period. In the clusters of pattern 1 (clusters 3, 6, 9, 10, 14), the number of cell clusters has decreased dramatically, and some have even totally disappeared in the day 11 sample (Fig. 1b). In contrast, the cells in the clusters of pattern 2 (clusters 0, 1, 2, 4, 5, 7, 8, 11, 12, 13 and 15), express essentially genes not or very poorly expressed in the clusters of pattern 1 (Fig. 1c). We attribute logically these changes to the influence of the host flies having received the injection of the OCs. This presupposes that the host has receptors to recognize the presence of the OCs (or their products) and, importantly, has the capacity to influence the destiny of these invading cells.

Gene Ontology Analysis of the 16 Clusters Identified above

We next analyzed the GO for each of the 16 clusters and report here the main information which we retained from this analysis (detailed data are presented in online suppl. Fig. 1–16; online suppl. Tables 2–17). The five clusters (3, 6, 9, 10, 14) of pattern 1 (day 0 sample) predominantly express genes coding for Ribosomal proteins, RNA-binding components, and translation factors. Further, within cluster 6, we note the expression of various genes related to axon guidance, nervous system development, imaginal wing disc morphogenesis, dorsal/ventral pattern formation, etc. These data are compatible with those published by Simcox et al. [18] and especially Dequéant et al. [19] regarding the origins of the RasV12 cell line.



5

(Figure continued on next page.)

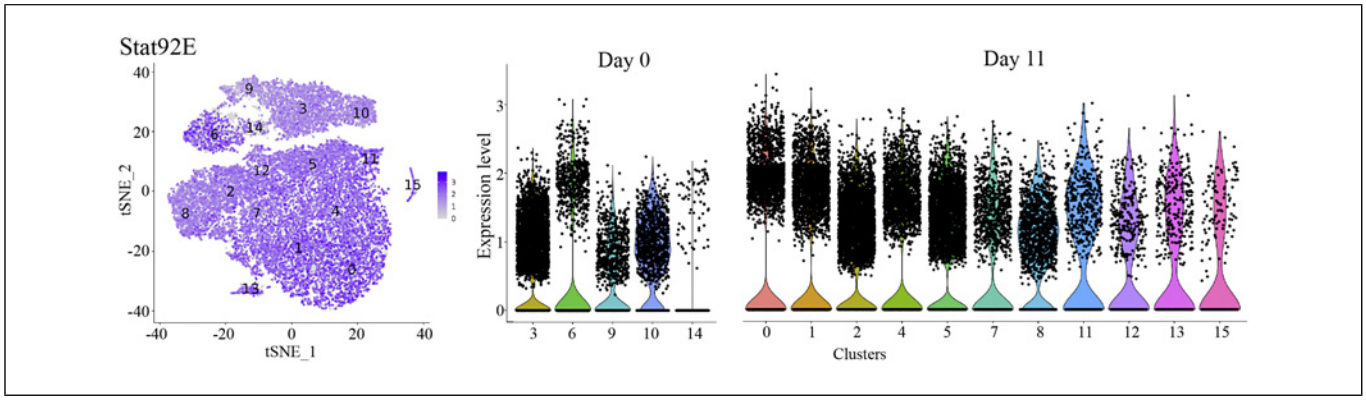


Fig. 5. Feature plots and violin plots for genes in JAK-STAT pathway for each cluster of day 0 and day 11 samples. Feature plot (picture on the right): the indicated gene expression on each cell was labeled with blue color dot. The relative expression level of each gene was indicated by the intensity of color blue. Cells will be labeled as gray color when their expression levels of indicated gene are non-detectable. Violin plots (picture on the left): the *Y* axis marked the expression level of the indicated genes. The *X* axis represents each cluster of day 0 sample and day 11 sample. The indicated gene expression on each cell was labeled with one black dot.

For pattern 2 of day 11, sample four clusters (0, 12, 13, and 15) account each for close to one-fifth of the total sample. As a reminder, these cells were not present as such at the time of injection of the OCs into the flies. GO analysis indicates that the cells in these clusters express predominantly genes coding for proteins involved in development, morphogenesis, and cell migration. The genes in cluster 2 are to a large extent coding for proteins involved in transmembrane transport, ATP biosynthesis, and similar basic aspects of cell physiology. The genes in cluster 4 rank among genes responsible for cell adhesion, invasion, and functions of membranes. The genes expressed in cluster 7 are mostly related to the production of energy (some similarity with cluster 2) and those of cluster 8 are related to cell cycle.

In contrast to the clusters analyzed above, the cells in cluster 1 and cluster 5 express genes which are frequently reported during inflammatory or defense reactions *sensu lato* in flies (e.g., receptors for phagocytosis and/or markers of plasmatocytes, the predominant blood cell type in *Drosophila*) [22]. Finally, the cells of cluster 11 (which represent only 2.39% of the genes in the day 11 sample) express similarly most genes encountered for clusters 1 and 5 and in addition low levels of some of the genes for AMPs, such as *Metchnikowin* (*Mtk*), *Cecropin C* (*CecC*), and *Drosomycin* (*Drs*), which are almost absent from the day 0 sample in our conditions.

Our previous study based on bulk transcriptomics of the day 0 and day 11 samples had led us to propose that GFP-positive cells isolated from day 11 flies contained cells that had acquired that capability of expressing markers of immune defenses [21]. This induced us here to deepen the analysis of the expressions of putative defense-related genes in day 11 sample by single cell sequencing in clusters 1, 5, and 11, representing roughly one-third of the day 11 sample. Nevertheless, an important caveat in this analysis is that we now know that a large proportion of the genes controlling defenses also play roles in developmental events (often in *Drosophila* at different developmental stages), and in several as yet poorly defined physiological processes.

In-Depth Analysis of Clusters Expressing Predominantly Genes Reported to Be Related to Inflammation and Defense Reactions

In the following section, we have visualized by feature plot analysis the expression levels of a series of genes which are considered to be potentially (albeit not exclusively) involved in reactions against various types of insults, namely infections, injuries, and cancer [8, 16]. At this stage of our knowledge, this list is obviously not exhaustive and the genes or gene families are ordered by simple convenience as they appeared during our experiments.

Toll Pathway

As apparent in the feature plot, the proportion of cells and the average expression level per cell of *spätzle* (*spz*) is markedly higher on day 11 than on day 0 (online suppl. Fig. 17) (Fig. 2). The Spätzle protein has been characterized as the activating ligand of the transmembrane receptor Toll and its massive presence here reflects probably the developmental role of the Toll pathway rather than its immune functions (we also keep in mind that the cell lines were

prepared from embryos). We further observed in the violin plots that most genes in the Toll pathway [23, 24], especially those encoding the receptor Toll, the adaptor Myd88 and the negative regulator kinase Cactus were abundantly expressed in most cells of both samples (day 0 and day 11), but their average expression levels/cell were higher on day 11. Of note, the average expression levels/cell of *Toll*, *Myd88*, *tube (tub)*, *dorsal (dL)*, *Dorsal-related immunity factor (Dif)*, are relatively high in cluster 6 (day 0 sample) and in clusters 0, 12, 13, and 15 (day 11 sample). Cells in those clusters abundantly express developmental markers, such as *lethal (2) giant larvae (l(2)gl)*, *scribble (scrib)*, *PDGF-*, and *VEGF-receptor related (Pvr)*, etc., suggesting also that these results reflect the developmental roles of the Toll pathway rather than its implication in defense. Of further interest, the proportion of cells expressing the two Toll pathway transcription factors *dl* and *Dif* is similar in both feature and violin plots, although *dl* appears to be more intensely expressed as regards the average expression level per cell.

IMD Pathway

In this pathway, the genes were abundantly expressed in most of the cells of the clusters 1, 5, and 11 of both day 0 and day 11 samples, but their expression levels/cell were relatively higher in the day 11 sample (Fig. 3). The transcription factor Relish [25] is expressed in almost all cells on both samples with a higher average expression level per cell in the day 11 sample. Although this pathway has been well documented as inducing several AMPs, we were not able here to monitor any detectable expression of those AMPs, except for *CecC*, *Mtk*, and *Drs*, which will be discussed later. To the best of our knowledge, the IMD pathway has not been reported to date to play a role in development.

JNK Pathway

Most genes of this pathway, especially *eiger (egr)*, *kayak (kay)*, and *hemipterous (hep)* are abundantly expressed in all cells of both samples, but the average expression levels/cell were higher in day 11 sample than in day 0 (online suppl. Fig. 17) (Fig. 4). In particular, kinases *hep* and *basket*, the transcription factors *Jun-related antigen (Jra)*, *kay* and *forkhead box, sub-group O* (*foxo)* were highly expressed in cluster 0 and 6, the cells of which extensively produce developmentally related markers. These data point to a developmental role of JNK pathway in our experiment here rather than to its possible role in immune defenses.

JAK-STAT Pathway

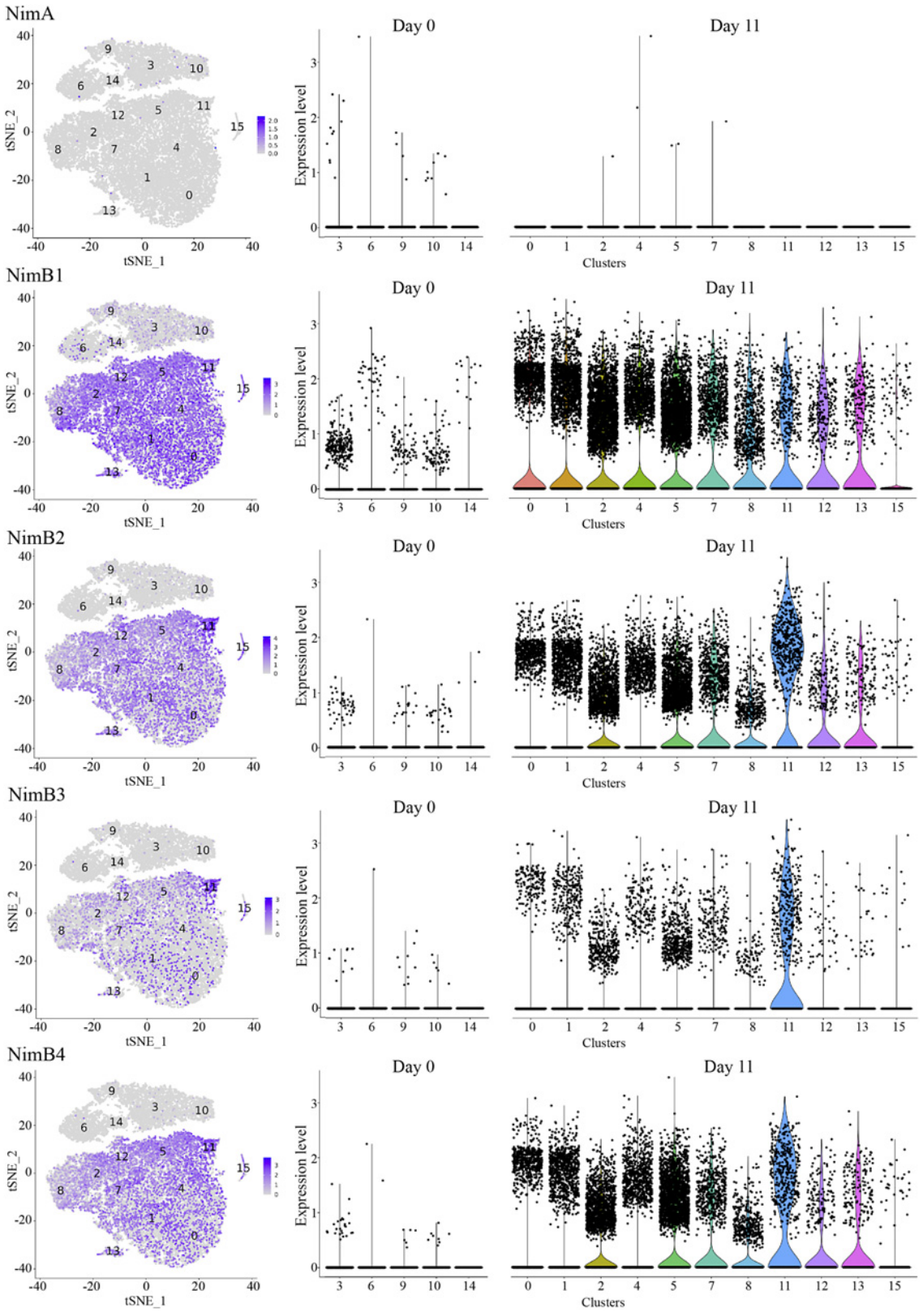
The gene encoding the receptor *domeless* (*dome)*, the adaptor *hopscotch (hop)*, and the transcription factor *Signal-transducer and activator of transcription protein at 92E* (*Stat92E)* were highly expressed in most cells in both samples, the expression levels/cell being especially high for *Stat92E*. Unlike for the *egr* gene (coding for the ligand of the JNK pathway), the genes encoding the ligands unpaired 1, 2, 3 of the JAK-STAT pathway were expressed in a relatively small proportion of cells and the average expression levels/cell was modest (Fig. 5). Finally, the expression levels/cell of *dome*, *hop*, and *Stat92E* were particularly high in cells of the developmentally related clusters 0 and 6. These data indicate that JAK-STAT pathway is constantly activated in RasV12 cells before and after injection into the flies and thus can exert an essential role in maintaining the RasV12 population.

Phagocytosis/Plasmatocytes

The expressions of the genes coding for proteins involved in phagocytosis were remarkably intense in day 11 sample. In particular, the percentage of cells expressing members of the Nimrod family [26] and their expression levels/cell were dramatically increased from the day 0 sample to day 11 sample, except for *Nimrod C4 (NimC4)*, which was already highly expressed in most cells of the day 0 sample (Fig. 6). In addition, *eater* [27] and *Scavenger receptor class C, type I* (*Sr-CI*) [28] share the same expression pattern with the Nimrod family members. The *peste* (*pes)* and *draper (drpr)* genes were strongly expressed in both the day 0 and the sample day 11. These data indicate that some cells of day 11 have developed strong phagocytic properties. It led us to investigate the markers of plasmatocytes, which are the primary phagocytes of *Drosophila* (evocative of the mammalian macrophage) and are involved in a variety of processes including defense and development [22]. As a first observation, except for the serine protease inhibitor *serpent (srp)* and for *croquemort (crq)*, we noted that all classical plasmatocyte markers, including *Peroxidasin (Pxn)*, *Hemolectin (Hml), eater*, *Nimrod C1 (NimC1)* (see also Fig. 6), and *Hemese (He)*, were expressed in a much larger proportion of cells in the day 11 sample than in the day 0 sample. In addition, their average expression levels/cell were increased sharply from day 0 to the day 11 sample (online suppl. Fig. 17). These data are strongly in favor of a prominent role of plasmatocytes in our experimental system, most certainly also through their phagocytic properties.

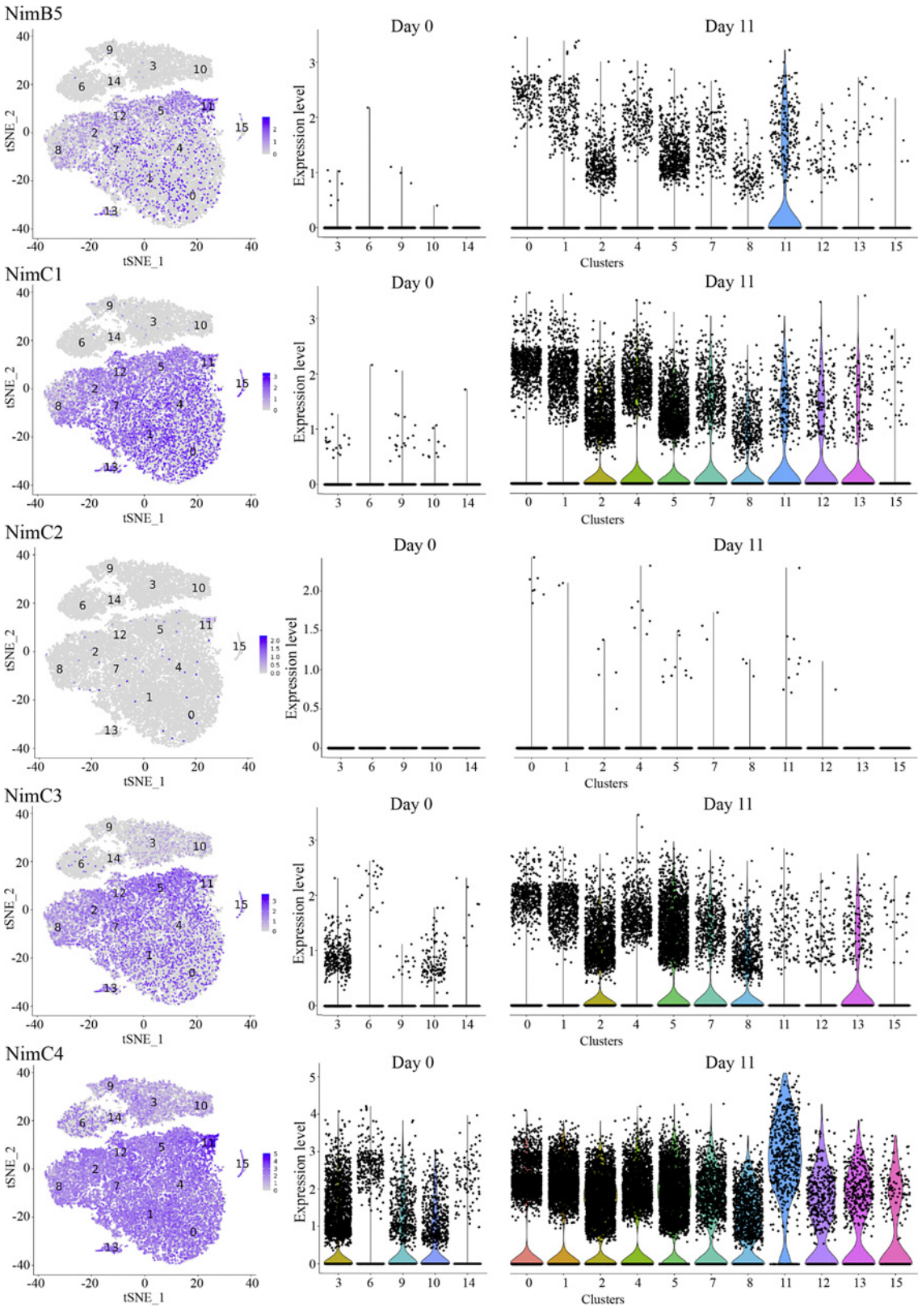
Antimicrobial Peptides

It is well established that in flies AMPs play a central role in the defense against a variety of bacteria and fungi (as by the way, in most whose expressions we recorded



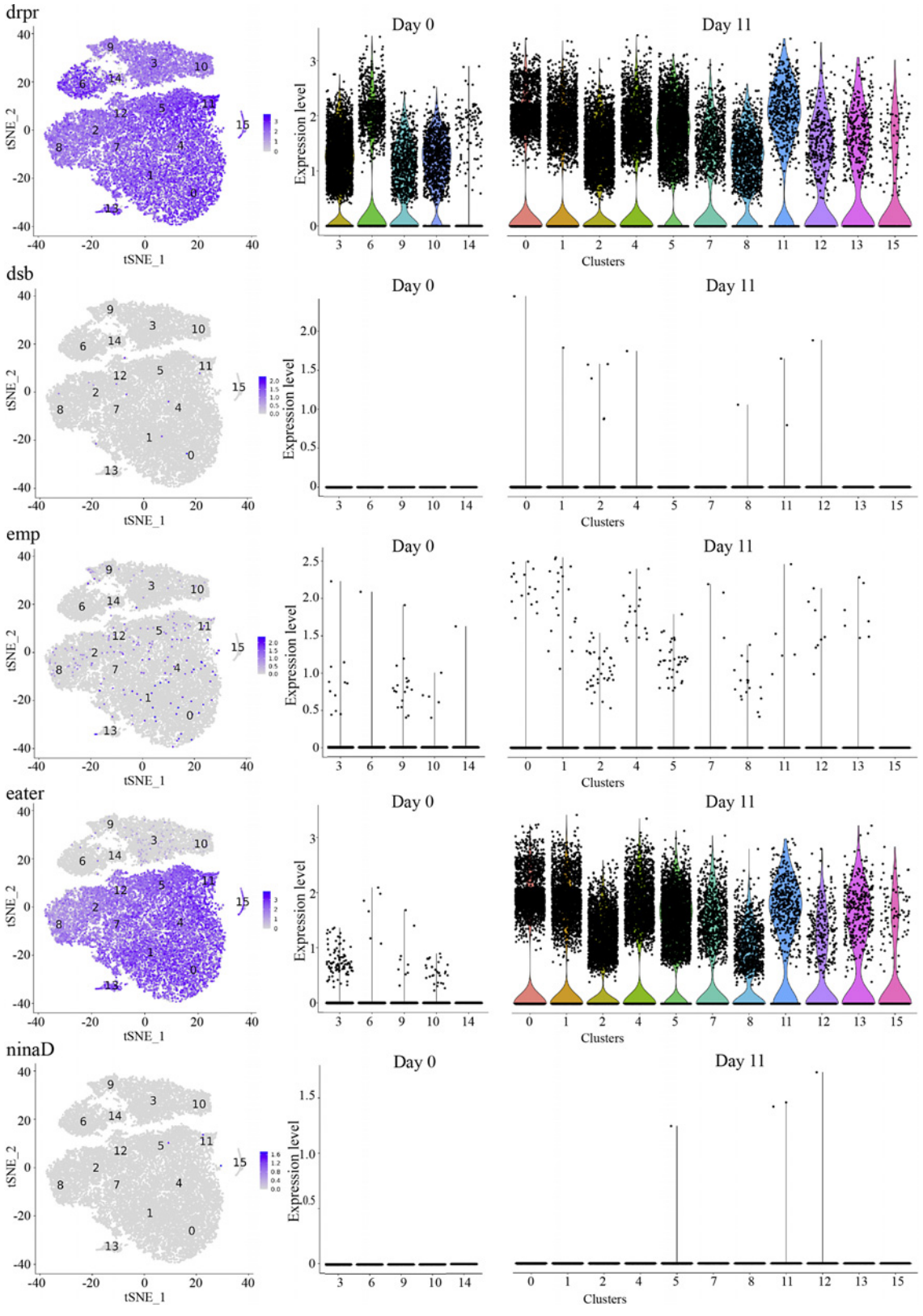
6

(Figure continued on next page.)



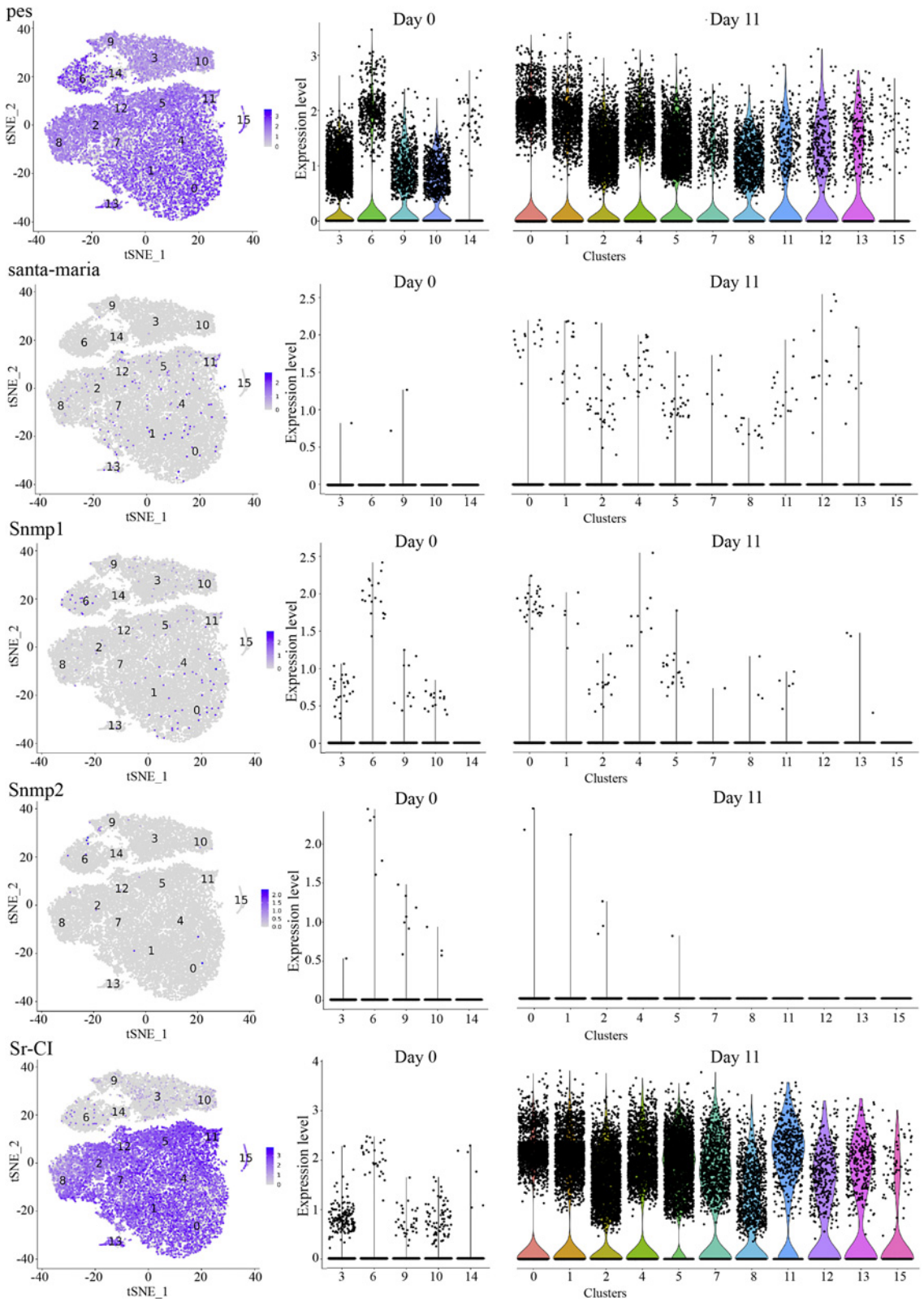
6

(Figure continued on next page.)



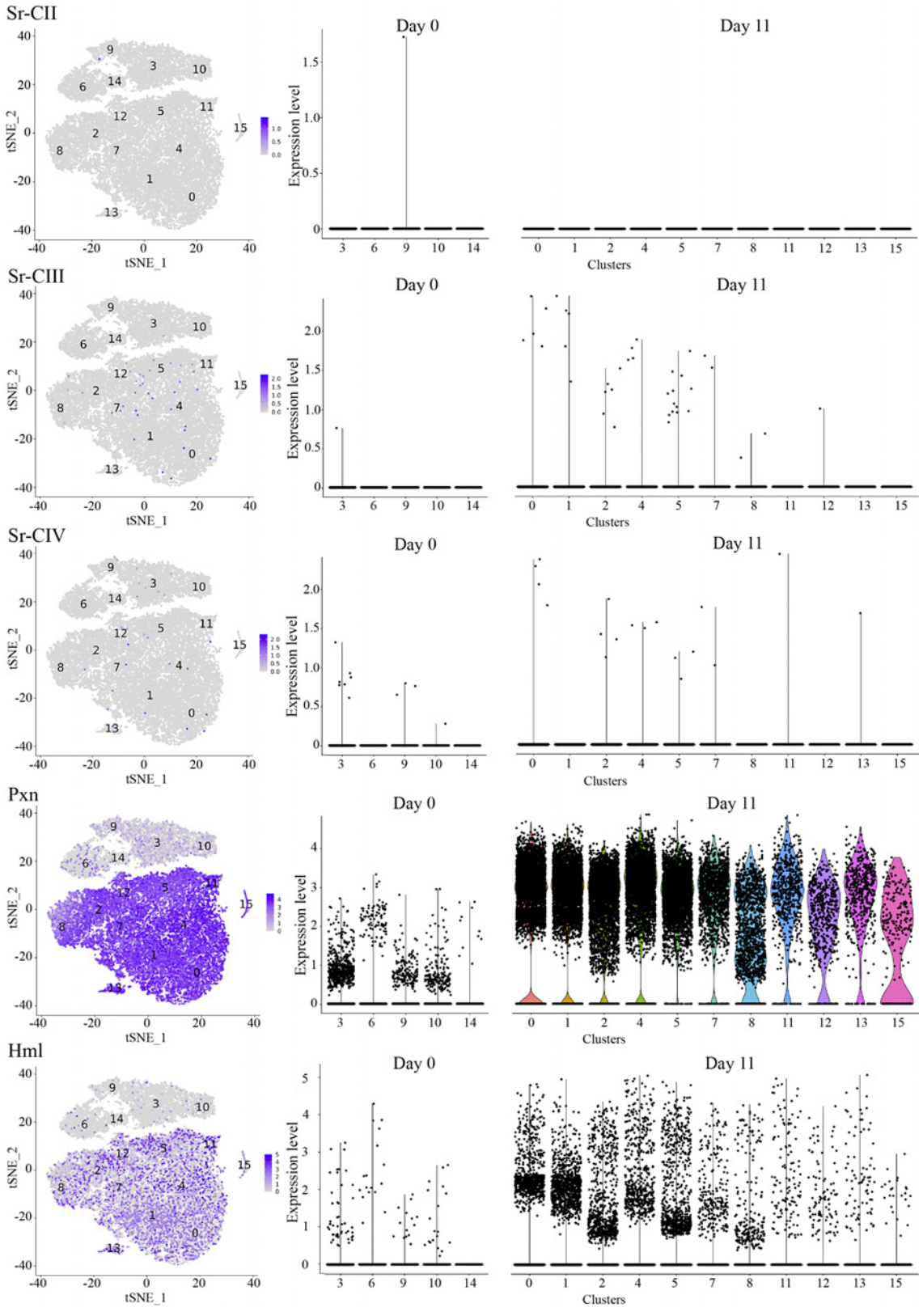
6

(Figure continued on next page.)



6

(Figure continued on next page.)



6

(Figure continued on next page.)

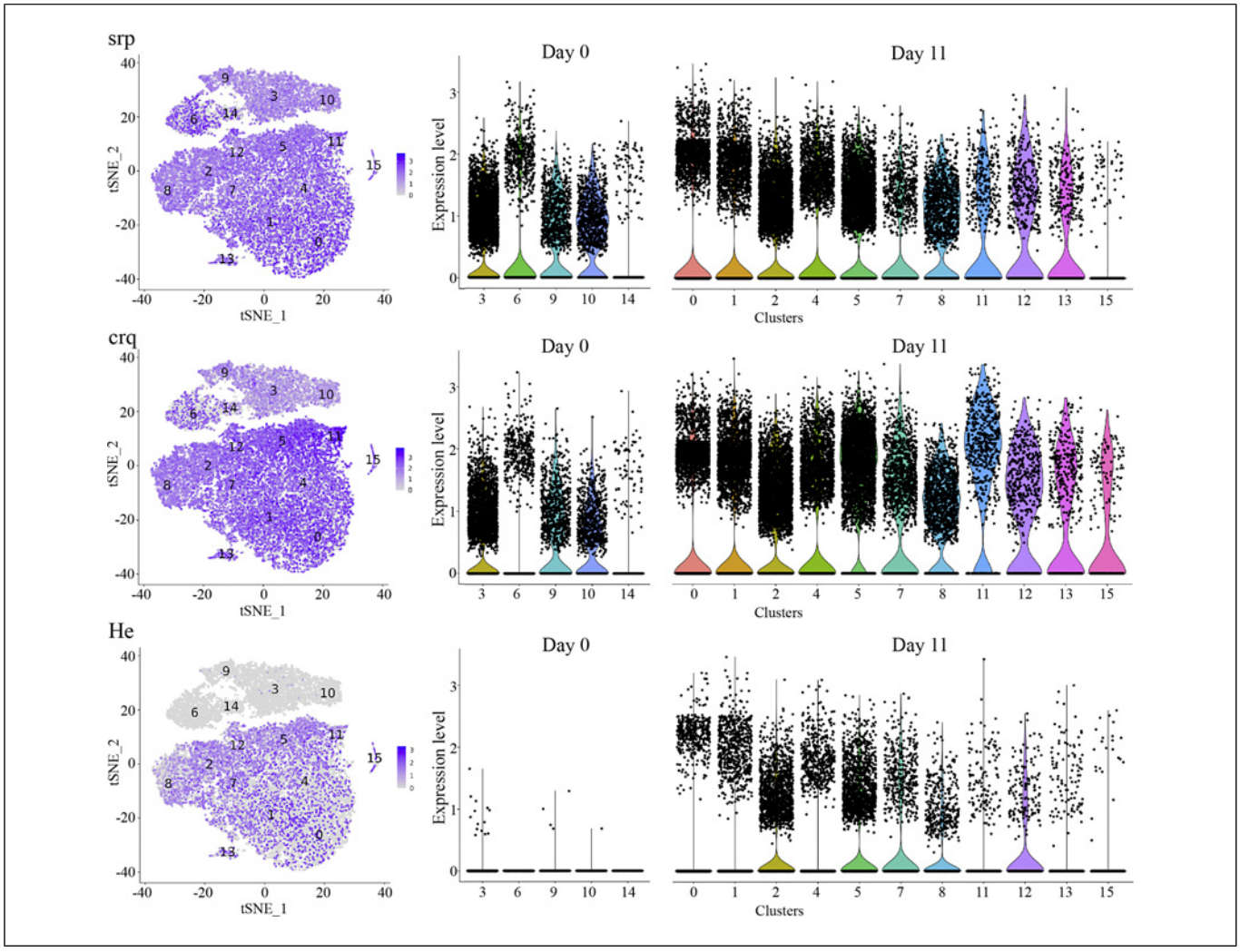
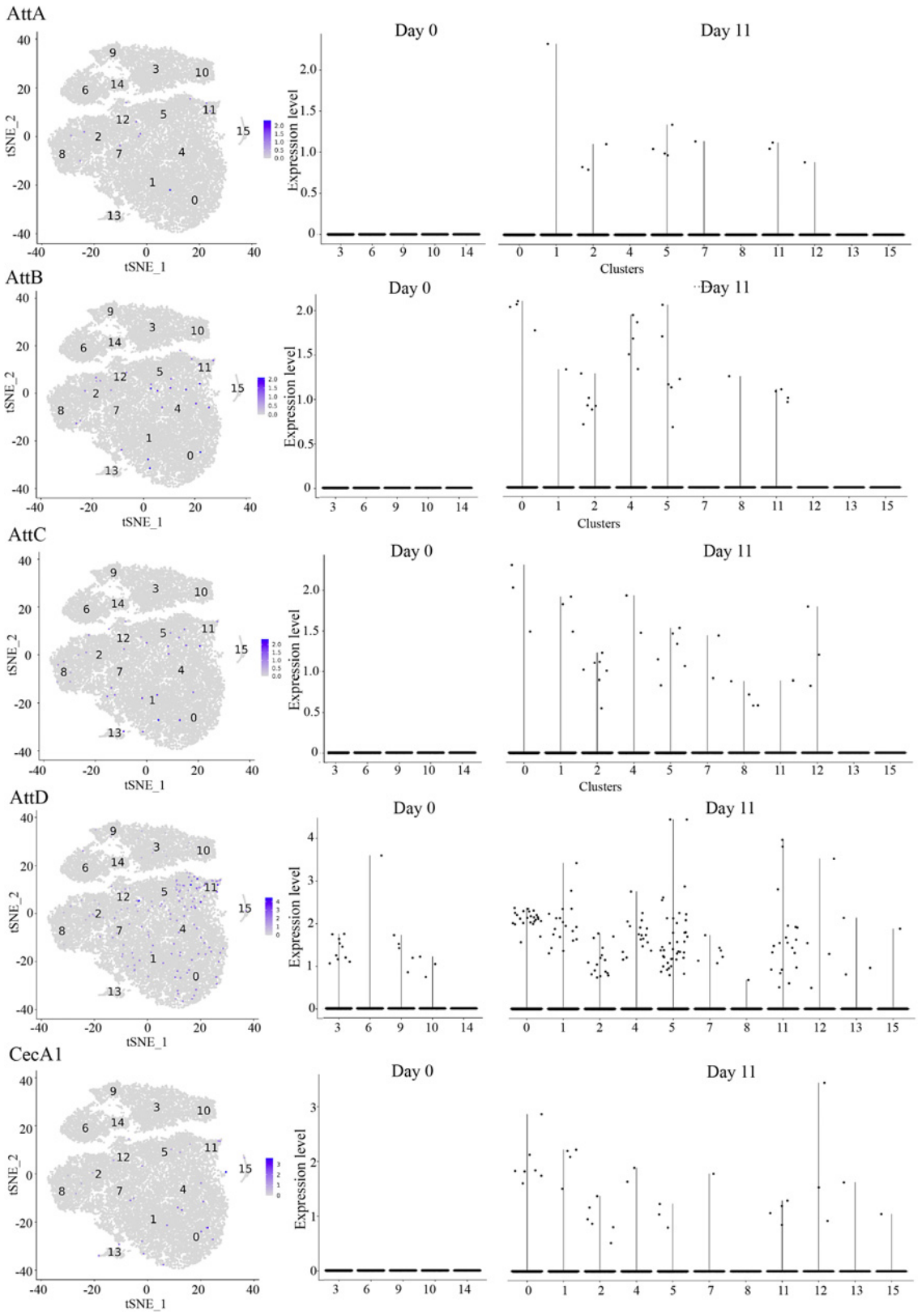


Fig. 6. Feature plots and violin plots for genes related to phagocytosis and plasmatocyte for each cluster of day 0 and day 11 sample. Feature plot (picture on the right): the indicated gene expression on each cell was labeled with blue color dot. The relative expression level of each gene was indicated by the intensity of color blue. Cells will be labeled as gray color when their expression levels of indicated gene are non-detectable. Violin plots (picture on the left): the *Y* axis marked the expression level of the indicated genes. The *X* axis represents each cluster of day 0 sample and day 11 sample. The indicated gene expression on each cell was labeled with one black dot.

animal and plant systems) [7] and early studies in the field have shown that the Toll and IMD pathways control separately or in concert the expression of these molecules (Fig. 7). It is significant for our present study that of the genes encoding 22 distinct AMPs which we addressed here, we were unable to note a marked expression of the genes encoding these peptides, with the modest exception of *Drs*, *Mtk*, and *CecC*, whose presence is essentially limited to clusters 1, 5, and 11 (that is the clusters which we identified above as expressing inflammation-related genes). Further, in day 0 samples, we did not observe a significant expression of any AMP genes with exception of the three genes noted above.

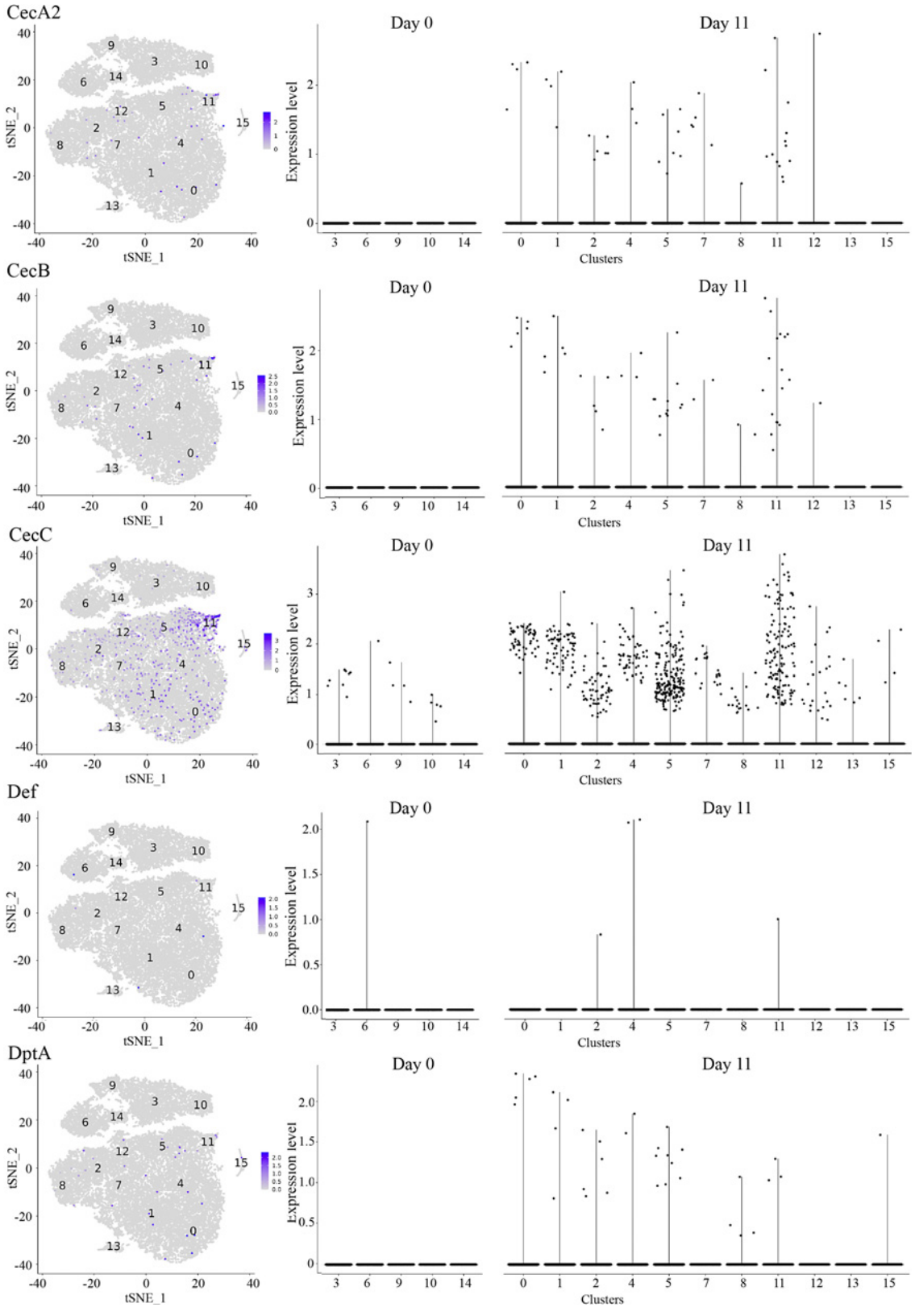
Pilot Experiments Validate the Relevance of Eater and Relish in the Early Stages of the Fy Response to the Injection of Oncogenic Cells

We hypothesized that genes expressed in a larger proportion of cells per sample and a higher level per cell in day 11 (as compared to day 0 samples), played a major role in the dialogue between the OCs and the host. We therefore performed a pilot functional experiment with



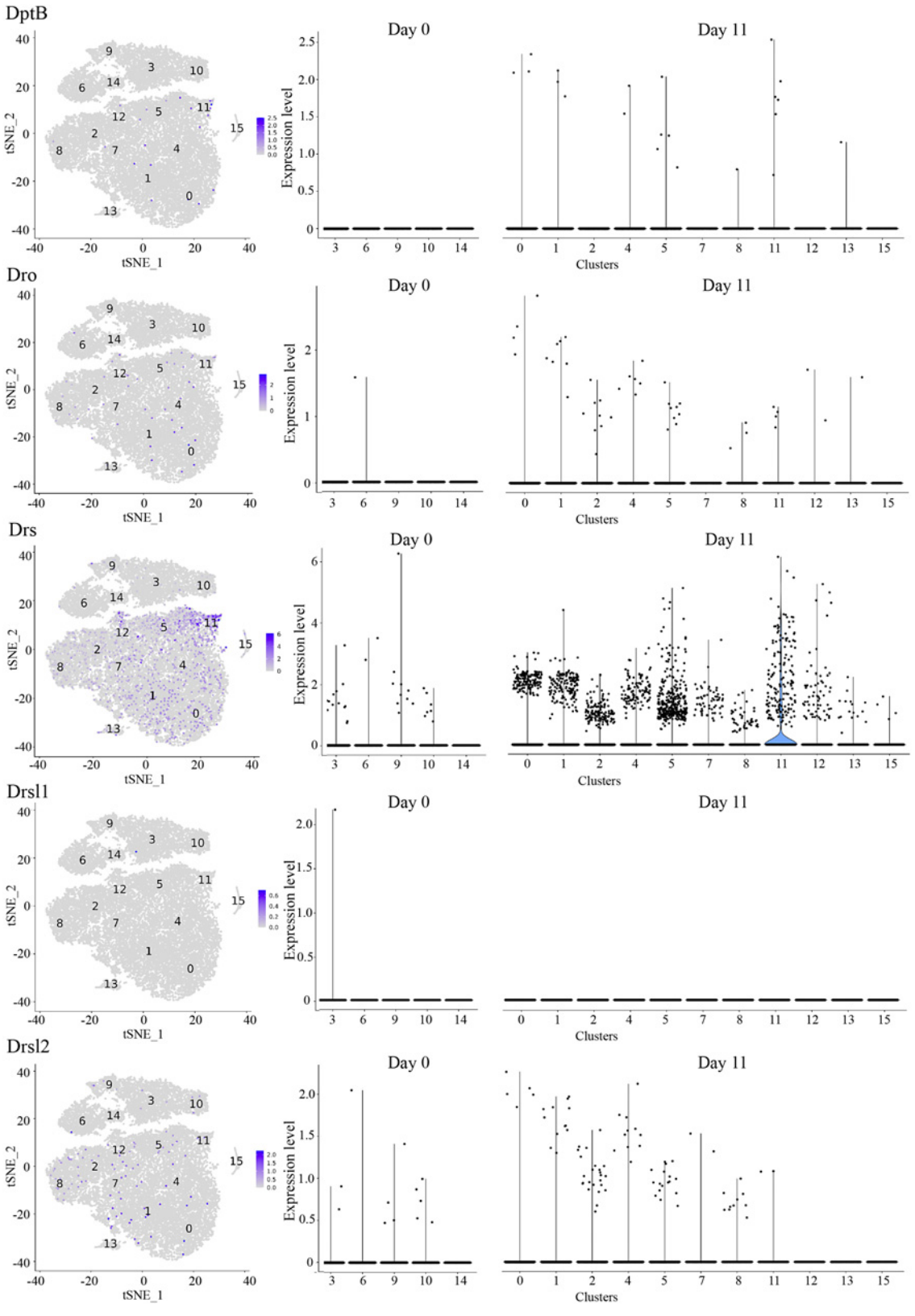
7

(Figure continued on next page.)



7

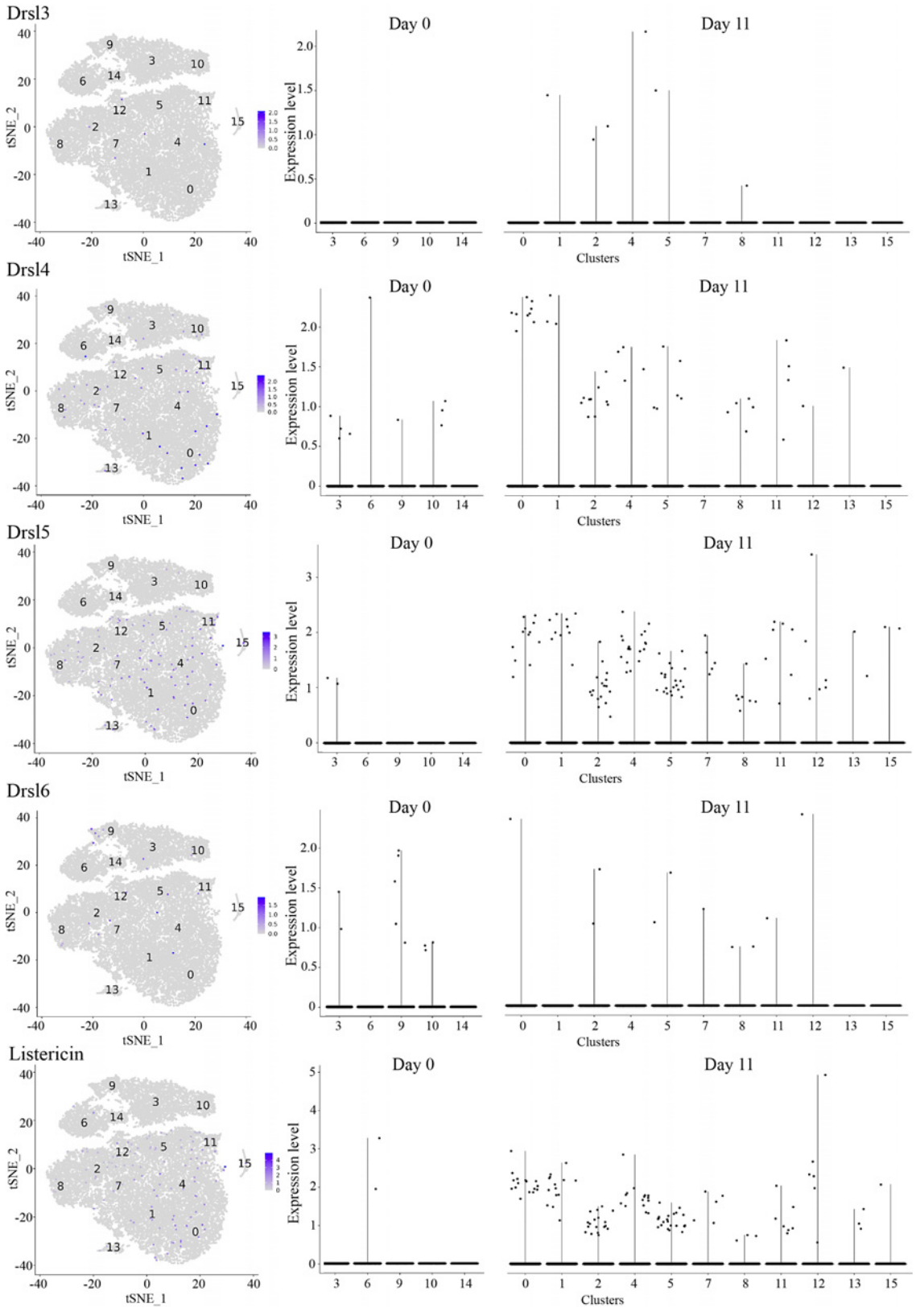
(Figure continued on next page.)



7

(Figure continued on next page.)

J Innate Immun 2023;15:442–467
DOI: 10.1159/000529096



7

(Figure continued on next page.)

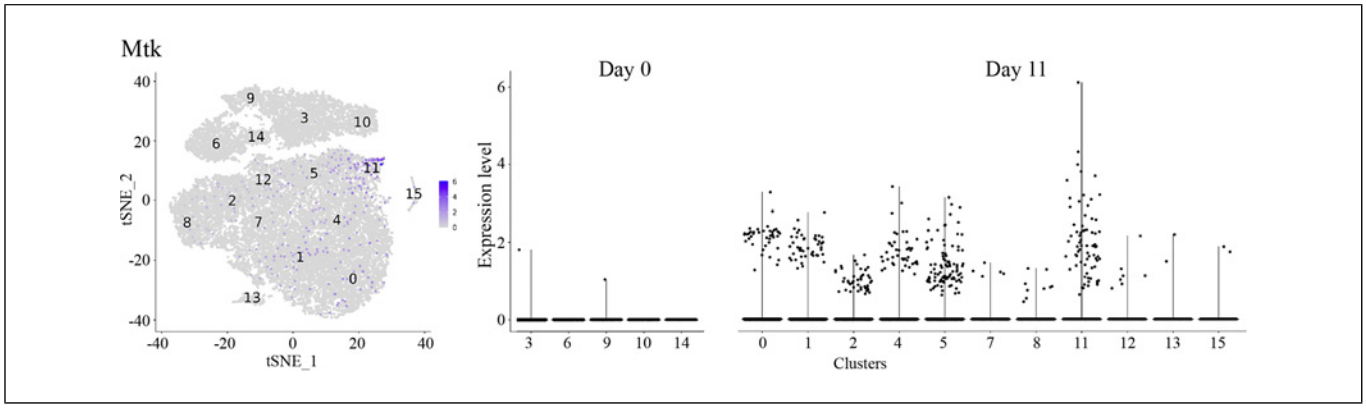


Fig. 7. Feature plots and violin plots for genes related to AMPs for each cluster of day 0 and day 11 sample. Feature plot (picture on the right): the indicated gene expression on each cell was labeled with blue color dot. The relative expression level of each gene was indicated by the intensity of color blue. Cells will be labeled as gray color when their expression levels of indicated gene are non-detectable. Violin plots (picture on the left): the *Y* axis marked the expression level of the indicated genes. The *X* axis represents each cluster of day 0 sample and day 11 sample. The indicated gene expression on each cell was labeled with one black dot.

the genes encoding *eater* and *Relish* which are both highly expressed in the OCs (see feature plot analyses presented in Fig. 2, 6). We silenced these genes by siRNA in the OCs of day 0 samples and (after monitoring the efficiency of the RNAi treatment) we injected them into day 0 adult male flies. As shown in Figure 8, silencing of *eater* in day 0 cells markedly affected the increase of the OC load, as compared to the controls (~60% lower values observed on day 3 postinjection). The silencing of *Relish* had a similar effect on the proliferation of the injected OCs which was reduced by 80% during the period investigated in these experiments, shown in Figure 8.

Discussion

Our objective was to follow the destiny of the cell groups within the flies after injections and to start a project of understanding the supposed dialogue between host and OCs in the same context. At this moment, for technical reasons, we were limited to compare the transcriptomic profiles of the cells at the time of injection into the host to cells which we could properly recover (by cell sorting based of the GFP marker) on day 11 only. Indeed, as we had previously established, the injected cells proliferate slowly after injection, reaching close to 5% of the total cells in the flies (flies containing proliferated OCs) 8 days after the onset of the experiments and they then exponentially grow to nearly 60% of the total cells on day 11 when the recipient flies have already massively died out (50% of the experimental population). This explains that we were unable to pin down precisely when the evolution/transformation of the injected cells in the flies started – this will have to be established in later experimental series. Notwithstanding this shortcoming, we may conclude from the present data that: (1) about one-third of the injected cells disappear as such during the experiments, (2) most of the other cells continue their development and express essentially genes related to this effect (we have not made a precise analysis of this group of cells which are not directly relevant to our present objectives); (3) a significant group of these cells (about one-third of the total on day 11) appears during their presence in the fly body to evolve their activities to a predominantly "inflammatory" phenotype. Among the latter, we noted a considerable level of expression of genes encoding both membrane or secreted phagocytic receptors and a discrete expression of a few (three, in this study) inducible AMPs; (4) in this same group of cells (as in many of the other cells of the day 11 sample (i.e., cells which have not disappeared as such after injection into the flies), we noted the expression of most of the genes characteristic of several signaling pathways, namely of the Toll and IMD pathways, as well as of the JNK and JAK-STAT pathways. As mentioned already, the Toll pathway is known for its roles in both development and defense, which is to various extents also the case for the JNK and JAK-STAT pathway. For the IMD pathway, there is only clear-cut evidence today for a role in defense, although we cannot exclude that such evidence may be found in the future. Obviously, understanding the roles of these pathways in our present system will require experimental studies to make any firm conclusions. That such studies can be informative is exemplified in Figure 8, where we could clearly show that reducing the expression levels of the *eater* transmembrane

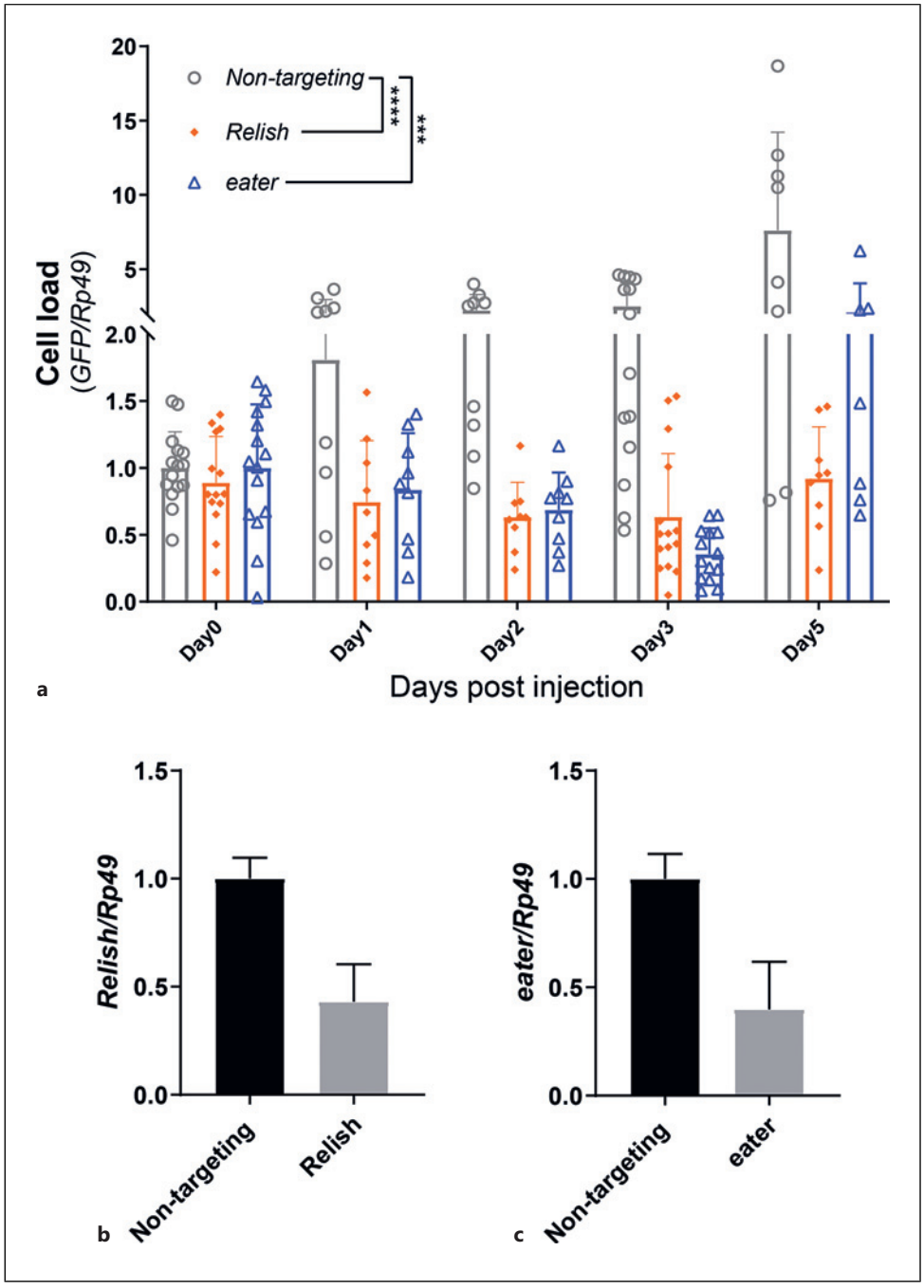


Fig. 8. The proliferation of *Relish* and *eater* knocked-down RasV12 oncogenic cells in W1118 wild-type *Drosophila* and the siRNA knock-down efficacy. **a** The proliferation of *Relish* and *eater* knocked-down RasV12 oncogenic cells in W1118 wild-type *Drosophila*. siRNAs targeting Relish and *eater* were transfected into RasV12 cells for 72 h, respectively. Non-targeting siRNA was used as control. After transfection, cells were harvested and injected into wild type W1118 *Drosophila* and the total RNA of injected flies was collected at indicated time point for RT-PCR. The cell load indicated by the relative expression of GFP mRNA after a normalization to the house-keeping gene ribosomal protein 49. The level of GFP expression on the day of injection (=day 0) was set as 1. One data point represents a pool of 6–8 flies for the cell load. The data points are collected from 4 independent experiments. Each experiment includes at least three bio-replicates. Two-way ANOVA was used for statistical analysis of the interactions between time course and fold change of cell load, $^{***}p < 0.001$. $^{****}p < 0.0001$. n.s. indicates statistically nonsignificant. **b**, **c** Silencing efficacy of *Relish* (**b**) and *eater* (**c**) in RasV12 oncogenic cells. The *Relish*-targeting siRNA was transfected into RasV12 oncogenic cells for 72 h. The non-targeting siRNA was used as control. After transfection, the total RNA of the transfected cells was collected and the expression level of *Relish* was detected by RT-PCR. The mRNA expression level of *Relish* was indicated after normalization to Rp49.

phagocytic receptor [27] or the *Relish* transcription factors [25] by RNA interference in the OCs prior to their injection into the flies reduced dramatically the rate of proliferation of these cells during the subsequent days. *Relish* is known as a *Drosophila* homolog of *NF-kB* in mammals, whose role in cancer development and progression through inflammation has been studied and discussed [29]. We are now extending this type of experiments to other strongly expressed and to non-secreted molecules (to avoid overlap with molecules secreted by the host) to gain a better understanding for the cell side of the host-OC dialogue, and in parallel, we are developing approaches to better isolate the host cells (overcoming the proper cell sorting difficulties at stages earlier than day 11, as explained in the Section above) to generate a better picture of the host-OCs during this experimental series.

Acknowledgment

We thank Prof. Eric Westhof for critical reading of this manuscript.

Statement of Ethics

An ethics statement was not required for this study type, no human or animal subjects or materials were used.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

This study was supported by the China Overseas Expertise Introduction Plan "111" (D18010), by Centre National de la Recherche Scientifique – Institut de Biologie Moléculaire et Cellulaire (CNRS-IBMC), the University of Strasbourg Institute for Advanced Study (to J.A.H.), by Sino-French Hoffmann Institute of Guangzhou Medical University, by Guangzhou Medical University Discipline Construction Funds (Basic Medicine) (JCXKJS2022A02). D.C. is supported by Natural Science Foundation of China (NSFC, 32200578), Science and Technology Program of Guangzhou (202102020090). Y.M. is supported by National Science and Technology Innovation 2030 Major Project of China (2022ZD0205700), Natural Science Foundation of China (NSFC, 81972701), and CAMS Innovation Fund for Medical Sciences (CIFMS; 2021-I2M-1-074, 2022-I2M-2-004). Z.M. is supported by National Key R&D Program of China (2021YFF1200900, 2021YFF1200903 to Z.M.).

Author Contributions

Jules A. Hoffmann and Di Chen: devised the project and prepared the writing; Xiao Lan, Di Chen, Xiaoming Huang, Jieqing Huang, and Xiaojing Zhou: performed the experiments and data analysis. Shanming Ji, Yuting Ma, Zhichao Miao, and Akira Goto: contributed to data analysis, discussions, and figure organization.

Data Availability Statement

The raw single-cell RNA-seq data generated during this study are available at the National Genomics Data Center (NGDC) under the accession number: PRJCA010962. Further inquiries can be directed to the corresponding author.

References

1 Steiner H, Hultmark D, Engström A, Bennich H, Boman HG. Sequence and specificity of two antibacterial proteins involved in insect immunity. Nature. 1981;292(5820):246–8.
2 Boman HG, Hultmark D. Cell-free immunity in insects. Annu Rev Microbiol. 1987;41:103–26.
3 Boman HG. Antibacterial peptides: key components needed in immunity. Cell. 1991;65(2):205–7.
4 Hoffmann JA. The immune response of Drosophila. Nature. 2003;426(6962):33–8.
5 Ferrandon D, Imler JL, Hetru C, Hoffmann JA. The Drosophila systemic immune response: sensing and signalling during bacterial and fungal infections. Nat Rev Immunol. 2007;7(11):862–74.
6 Lemaitre B, Hoffmann J. The host defense of Drosophila melanogaster. Annu Rev Immunol. 2007;25:697–743.
7 Boman HG. Antibacterial peptides: basic facts and emerging concepts. J Intern Med. 2003;254(3):197–215.
8 Hanson MA, Lemaitre B. New insights on Drosophila antimicrobial peptide function in host defense and beyond. Curr Opin Immunol. 2020;62:22–30.
9 Hoffmann JA, Kafatos FC, Janeway CA, Ezekowitz RA. Phylogenetic perspectives in innate immunity. Science. 1999;284(5418):1313–8.
10 Stark MB. An hereditary tumor in the fruit fly, Drosophila. Cancer Res. 1918;3(3):279–301.
11 Gateff E. The genetics and epigenetics of neoplasms in Drosophila. Biol Rev Camb Philos Soc. 1978;53(1):123–68.
12 Gateff E. Cancer, genes, and development: the Drosophila case. Adv Cancer Res. 1982;37:33–74.
13 Gonzalez C. Drosophila melanogaster: a model and a tool to investigate malignancy and identify new therapeutics. Nat Rev Cancer. 2013;13(3):172–83.
14 McGranahan N, Swanton C. Clonal heterogeneity and tumor evolution: past, present, and the future. Cell. 2017;168(4):613–28.
15 Enomoto M, Siow C, Igaki T. Drosophila as a cancer model. Adv Exp Med Biol. 2018;1076:173–94.
16 Urban-Wojciuk Z, Khan MM, Oyler BL, Fahraeus R, Marek-Trzonkowska N, Nita-Lazar A, et al. The role of TLRs in anti-cancer immunity and tumor rejection. Front Immunol. 2019;10:2388.
17 Villegas SN. One hundred years of Drosophila cancer research: no longer in solitude. Dis Model Mech. 2019;12(4):dmm039032.

18 Simcox A, Mitra S, Truesdell S, Paul L, Chen T, Butchar JP, et al. Efficient genetic method for establishing Drosophila cell lines unlocks the potential to create lines of specific genotypes. PLoS Genet. 2008;4(8):e1000142.
19 Dequeant ML, Fagegaltier D, Hu Y, Spirohn K, Simcox A, Hannon GJ, et al. Discovery of progenitor cell signatures by time-series synexpression analysis during Drosophila embryonic cell immortalization. Proc Natl Acad Sci U S A. 2015;112(42):12974–9.
20 Chen D, Roychowdhury-Sinha A, Prakash P, Lan X, Fan W, Goto A, et al. A time course transcriptomic analysis of host and injected oncogenic cells reveals new aspects of Drosophila immune defenses. Proc Natl Acad Sci U S A. 2021;118(12):e2100825118.
21 Irving P, Troxler L, Heuer TS, Belvin M, Kopczynski C, Reichhart JM, et al. A genome-wide analysis of immune responses in Drosophila. Proc Natl Acad Sci U S A. 2001;98(26):15119–24.
22 Cattenoz PB, Sakr R, Pavlidaki A, Delaporte C, Riba A, Molina N, et al. Temporal specificity and heterogeneity of Drosophila immune cells. EMBO J. 2020;39(12):e104486.
23 Belvin MP, Anderson KV. A conserved signaling pathway: the Drosophila toll-dorsal pathway. Annu Rev Cell Dev Biol. 1996;12:393–416.
24 Nusslein-Volhard C. The toll gene in Drosophila pattern formation. Trends Genet. 2022;38(3):231–45.
25 Hedengren M, Asling B, Dushay MS, Ando I, Ekengren S, Wihlborg M, et al. Relish, a central factor in the control of humoral but not cellular immunity in Drosophila. Mol Cell. 1999;4(5):827–37.
26 Kurucz E, Markus R, Zsamboki J, Folkl-Medzihradszky K, Darula Z, Vilmos P, et al. Nimrod, a putative phagocytosis receptor with EGF repeats in Drosophila plasmatocytes. Curr Biol. 2007;17(7):649–54.
27 Kocks C, Cho JH, Nehme N, Ulvila J, Pearson AM, Meister M, et al. Eater, a transmembrane protein mediating phagocytosis of bacterial pathogens in Drosophila. Cell. 2005;123(2):335–46.
28 Rämet M, Pearson A, Manfruelli P, Li X, Koziel H, Göbel V, et al. Drosophila scavenger receptor CI is a pattern recognition receptor for bacteria. Immunity. 2001;15(6):1027–38.
29 Karin M. Nuclear factor-κB in cancer development and progression. Nature. 2006;441(7092):431–6.