Dynamics of Innate Immune Response in Bacteria-Induced Mouse Model of Pulpitis

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ABSTRACT

Introduction: During pulpitis, as bacteria penetrate deeper into the dentin and pulp tissue, a pulpal innate immune response is initiated. However, the kinetics of the immune response, how this relates to bacterial infiltration during pulpitis and an understanding of the types of immune cells in the pulp is limited. **Methods:** Dental pulp exposure in the molars of mice was used as an animal model of pulpitis. To investigate the kinetics of immune response, pulp tissue was collected from permanent molars at different time points after injury (baseline, day 1, and day 7). Flow cytometry analysis of CD45+ leukocytes, including macrophages, neutrophils monocytes, and T cells, was performed. 16S *in situ* hybridization captured bacterial invasion of the pulp, and immunohistochemistry for F4/80 investigated spatial and morphological changes of macrophages during pulpitis. Data were analyzed using two-way ANOVA with Tukey's multiple comparisons. **Results:** Bacteria mostly remained close to the injury site, with some expansion towards noninjured pulp horns. We found that F4/80^{+} macrophages were the primary immune cell population in the healthy pulp. Upon injury, CD11b $^{+}$ Ly6G^{high} neutrophils and CD11b $^{+}$ Ly6G^{int}Ly6C^{int} monocytes constituted 70-90% of all immune populations up to 7 days after injury. Even though there was a slight increase in T cells at day 7, myeloid cells remained the main drivers of the immune response during the seven-day time period. **Conclusions:** As bacteria proliferate within the pulp chamber, innate immune cells, including macrophages, neutrophils, and monocytes, predominate as the major immune populations, with some signs of transitioning to an adaptive immune response. *(J Endod 2023;49:1529–1536.)*

KEY WORDS

Pulpitis; innate immunity; neutrophil; macrophage; flow cytometry; animal model

SIGNIFICANCE

Resident macrophages alongside recruited neutrophils and monocytes dominated the pulp tissue in a bacteria-induced mouse model of pulpitis. Investigating the role and the dynamic changes of innate immune response in pulp pathology can inform and guide reliable and predictable vital pulp therapies.

Nonsurgical root canal therapy is a successful and highly predictable treatment for treating dental pulp inflammation and infection[1]. However, evidence shows that more conservative treatments, such as pulp capping and pulpotomy, can serve as successful treatment options and allow the reversal of tissue damage[2-4]. There are various challenges and gaps in knowledge for clinicians to provide predictable conservative treatments reliably. One key area where there is limited understanding is the dynamics of the immune response and how this correlates with tissue damage and bacterial progression within the coronal pulp tissue.

In the pulp, resident macrophages have been shown as the primary resident immune cell type that provide the surveillance of the healthy pulp tissue[5,6]. However, in the context of infection and pulpitis, less is known about the kinetics of the immune response, mainly because the analysis of these changes is technically challenging[7]. Myeloid immune cells, which include macrophages, neutrophils, and monocytes, are crucial members of innate immunity. Neutrophils and monocytes are circulating leukocytes that play a critical role in antibacterial host defense and are one of the first innate immune cells recruited to the site of infection or injury[8-10]. Neutrophils ingest and phagocytose bacteria, and their entry into tissues and subsequent cell death sets the stage for the resolution phase of the inflammation or a successful transition to an adaptive immune response[11,12]. Dysregulation of neutrophil recruitment and function can contribute to continuous tissue damage, as seen in some chronic inflammatory diseases[13,14]. Previous studies have also investigated the possible destructive role of neutrophils in the pulp tissue[15,16]. Understanding the dynamics of these immune cell populations can provide insight into the protective and destructive processes occurring during pulpitis[17,18].

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We are pleased to share the data upon request.

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Past studies examining human pulp tissue samples have investigated how the innate and adaptive immune response may relate to microbiome changes in dental caries[19-22]. However, these studies can only capture one-time point, rather than the dynamics of the response, and are limited to human samples collected once the disease has progressed significantly that the teeth require extraction or root canal treatment. Therefore, studying the innate immune response, which occurs at the initial stages of pulpal inflammation, is challenging in human tissues.

Using animal models of pulpitis would enable a better capture of the dynamics of the cellular response. A previous study using an injury model of mouse incisors showed a significant increase in the percentage of myeloid cells, which almost returned to baseline 3 days after injury[18]. However, murine incisor teeth grow continuously even after the teeth complete their maturation[6]. Therefore, it is possible that the immune response of continuously growing mice incisor teeth would differ from that of human teeth. In this study, we aimed to use a mouse model of pulpitis based on molar tooth injury, which can more closely resemble human pulpitis.

The aim of the study was to perform a detailed investigation of the kinetics of the immune cell response during pulpitis in mice molars by using flow cytometry of the pulp. To our knowledge, this is the first study that investigated the kinetics of innate immune response during pulpitis using flow cytometry in a mouse model of pulpitis using molar teeth. We also aimed to correlate the kinetics of the immune response with the bacterial progression in the pulp during the same time course. Spatially and kinetically, we found that macrophages were the resident immune cells, and they showed morphological changes that corresponded with the timeline of bacterial invasion. We also found that neutrophils and monocytes were the primary innate immune cell populations that entered the pulp and persisted until the later stages of the disease.

MATERIALS AND METHODS

Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) (IS00002576-3). C57BL/6 mice were purchased from an independent nonprofit biomedical research institution. All mice were housed, cared for by qualified veterinarians and veterinary technicians, and monitored for pain and postoperative distress in accordance with Institutional Animal Care and Use Committee (IACUC) standards in an American Association for Accreditation of Laboratory Animal Care accredited facility. Age-matched 7- to 12-week-old littermate mice of both sexes were used for experiments.

Dental Pulp Injury Procedure

Mice were anesthetized via intraperitoneal injection of 100 mg/kg ketamine + 10 mg/kg xylazine in sterile phosphate-buffered saline (PBS). The exposures were generated in accordance with published protocols[23,24]. Briefly, the molar tooth was drilled with a $^1/_4$ round bur at low speed until the pulp was exposed. The contralateral molar served as the uninjured control. For histology experiments, only the maxillary first molar for each animal was injured, and 4-5 animals were included in each group in each experiment[25]. For flow cytometry experiments, maxillary first, second, and mandibular first molars were injured in each animal, and teeth from 5 mice were pooled for one sample. The number of animals was determined based on pilot experiments conducted by pooling 10-15 molar teeth to have 60,000-100,000 events in flow cytometry. At least three replicated experiments were run for each group. For histological analysis, mice were euthanized by trans-cardially perfusion under deep anesthesia. For flow cytometry experiments, mice were euthanized by carbon dioxide inhalation followed by cervical dislocation. Both methods were performed in compliance with the American Veterinary Medical Association and were approved by IACUC.

Pulp Tissue Cell Suspension Preparation

Maxillae/and mandibles were dissected and put in minimum essential medium (MEM-α) on ice. Surrounding tissues of teeth, including gingiva, were removed. The crowns of teeth were separated using a scalpel blade at crown-root interference, which allowed both for exposure and collection of coronal pulp tissue by avoiding contamination from surrounding tissues. The crowns were placed into the MEM-α culture media. Culture media was discarded, and the crowns were incubated in a digestive solution (4 mg/ml collagenase II and 4 mg/ml dispase II) at 37°C for 1 hour. Later, the crowns within the digestive solution were pipetted vigorously for the pulp tissue to be detached from dentin, and the samples were filtered using 70 μm mesh. The pulp samples were spun down at 300g for 5 minutes and washed with flow buffer (2% FBS, 1 μM EDTA, 0.1% sodium azide).

Flow Cytometry

Cell suspensions were spun down at 300g for 5 minutes and re-suspended in a flow buffer. The cell suspension was incubated with FcR blocking reagent for ten minutes, washed with flow buffer, and then incubated with the following antibodies Zombi Aqua -KO525 (1:1000, Biolegend), CD45-Cy7 (1:100, Biolegend), CD11b-PE (1:100, Biolegend), Ly6G-APC (1:100, Biolegend), Ly6C-AF700 (1:100, Biolegend), CD3-FITC (1:100, Biolegend), CD4-PE-cy7 (1:100, Biolegend), CD8-perpCy5.5 (1:100, Biolegend), F4/80 PB (1:100, Biolegend) for 30 minutes. The cell suspensions were washed again, centrifuged, resuspended in 1% paraformaldehyde (PFA), and filtered through a 40 μm mesh. Counting beads (Biolegend) were added to each sample. Flow cytometry was performed on a CytoFLEX flow cytometer (Beckman Coulter Life Sciences), and data were analyzed using FlowJo software (FlowJo LLC).

Tissue Processing & Immunofluorescence Staining

Animals were perfused with PBS with heparin, followed by 4% PFA. Maxillae were dissected and cut out to separate right (injured tooth side) and left (noninjured tooth side) half maxillae. All tissues were postfixed with 4% PFA. Decalcification of maxillae using 18% EDTA for 3-4 weeks at 4°C was performed. Half the maxillae were dehydrated by 30% sucrose for 2 days, followed by 50% sucrose for 1 day at 4°C, and then cryopreserved. 30 μm cryosections were obtained. Cryosections were washed with PBS for 30 minutes. Sections were blocked with 5% goat serum and 1% triton for 1 hour at room temperature. Sections then were incubated with the primary antibody -rat anti-F4/80 (1:200, abcam)- overnight at 4°C. Sections were washed with 1% goat serum and 1% triton for 30 minutes and incubated with the secondary antibody -anti-rat Alexa fluor647 (1:500, ThermoFisher)- for 2 hours. Stained slides were mounted. Image acquisition was performed by confocal microscope (Leica, Stellaris). An equal number of stacks were imaged using the same acquisition settings. Image processing was performed using Fiji[26] with the same settings.

Fluorescence *In-situ* Hybridization

Cryosections were washed with PBS for 30 minutes. Half of the sections on one slide were incubated with a 1:200 Cy3-conjugated nonsense eubacterial probe (5′-/5Cy3/ CGACGGAGGGCATCCTCA-3′) whereas the other half of the sections on the same slide were incubated with 1:200 Cy3-conjugated

16S eubacterial probe (5′-/5Cy3/GCTGCCTCCCGTAGGAGT-3′) in 20% SDS, 1:10 formamide, and 9:10 bacterial hybridization buffer overnight in 50°C in hybridization oven. Sections were washed first with a hybridization buffer, followed by PBS before mounting. Image acquisition and processing were performed similarly with the immunofluorescence staining.

Statistical Analysis

Animals were randomly assigned, and researchers were blinded to experimental groups during data analysis. Statistical comparisons of three or more groups with one-time point were analyzed by one-way ANOVA with Tukey post-tests. Statistical comparisons of three or more groups with multiple time points were analyzed by two-way ANOVA with Tukey post-tests. Between-subject effects were used to determine if there had been any significant effects of time, experimental group, or a significant interaction, followed by posthoc tests where necessary. A *P*-value less than 0.05 was considered statistically significant. Statistical analyses were conducted using Prism 9 (GraphPad Software, LLC) and Excel (Microsoft).

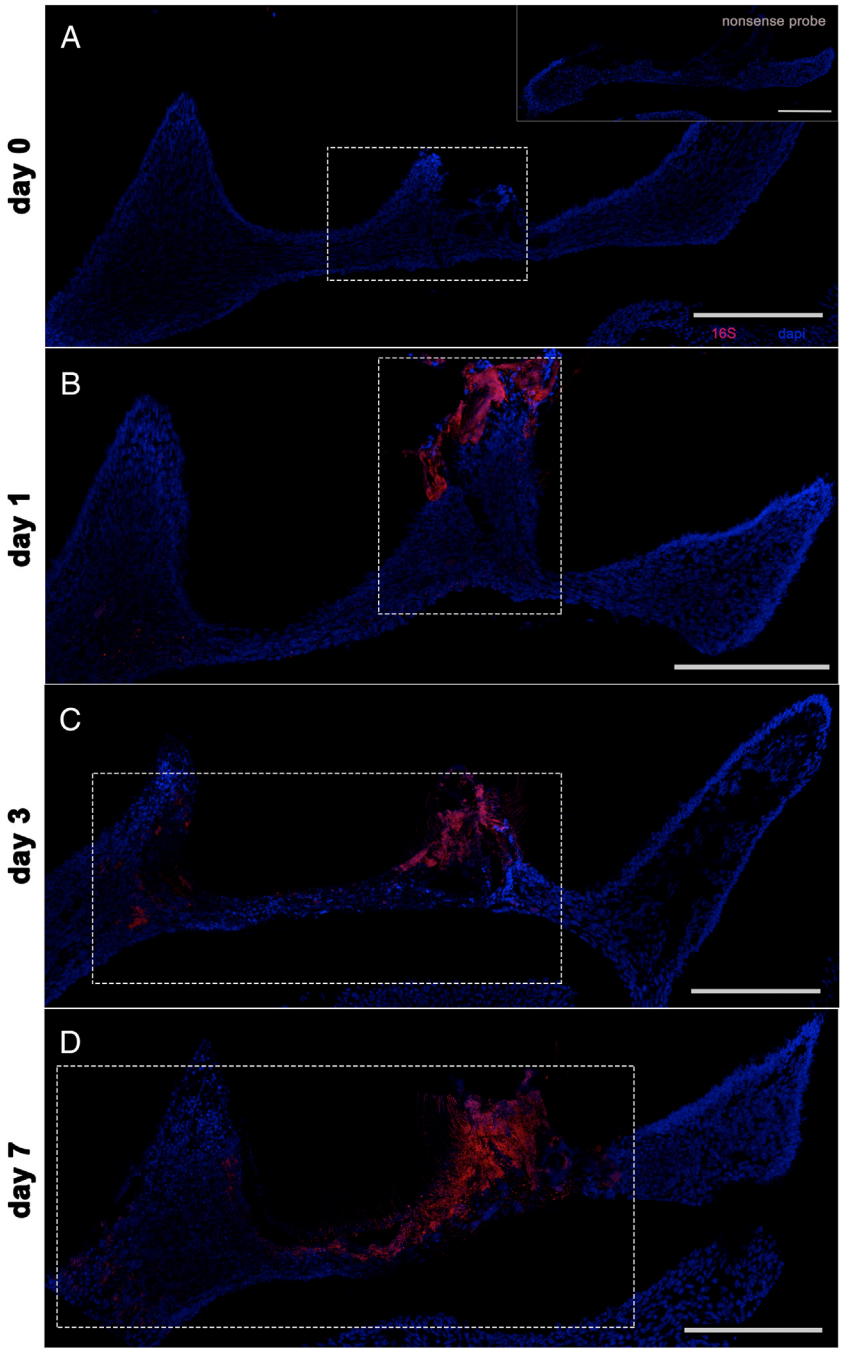

FIGURE 1 – 16S florescence-in situ hybridization to investigate bacteria localization within the pulp chamber across different time points. *A*, Pulp, just right after the injury. The *dashed box* shows the mechanically injured middle pulp with no bacteria present. The image in the top right corner is a section where the nonsense probe was used as a negative control. *B*, Pulp, one day after injury. The bacteria are localized at the injury site, shown within box. *C*, *D*, Pulp, 3 and 7 days after injury. The *boxes* show that most bacteria remain at the injury site but extend to the noninjured pulp horn. Scale bars are 200 μm. Red-16S probe, blue-dapi. Magnification: 20X.

RESULTS

Bacteria Remain within the Coronal Pulp Tissue up to Day Seven during Pulpitis

We wanted to observe bacteria penetration into the pulp space after pulp exposure over the same time frame we captured the immune response. By performing fluorescence *in situ* hybridization (FISH) of 16S bacterial rRNA in tissue sections, we found that at day 0, just after the injury, no bacteria were present at the injury site (Fig. 1*A*). On day 1 post-injury, bacteria were abundantly present at the injury site but only superficially located at the injured middle pulp horn (Fig. 1*B*). By days 3 and 7, most bacteria were still densely present close to the injury site. However, we also observed bacteria spreading to noninjured pulp horns and even at the coronal third of the root pulp (Fig. 1*C* and *D*).

Immune Cell Populations of the Healthy Pulp Tissue

We first wanted to study immune surveillance of healthy pulp tissue. Histologically, the F4/80+ macrophage population was densely present in the healthy pulp tissue (Fig. 2*A*). They exhibited an amoeboid cell shape with numerous cytoplasmic extensions (Fig. 2*A*). We next performed flow cytometric analysis of different subsets of CD45+ immune cells in the healthy dental pulp, including macrophages, neutrophils, monocytes, and T cells. In healthy intact pulp tissue, CD45+ immune cells constituted, on average, only 3% of all viable cells (Fig. 3*A* and *F*). CD11b + myeloid cells were the major immune cell population (74%) present, whereas only about 2.6% of resident immune cells were CD3+ T cells (Fig. 3*A*, *C*, and *H*). Of CD11b + myeloid cells, F4/80+ macrophages were the main immune cell population, forming, on average, 68% of all immune cells (Fig. 3*D* and *H*).

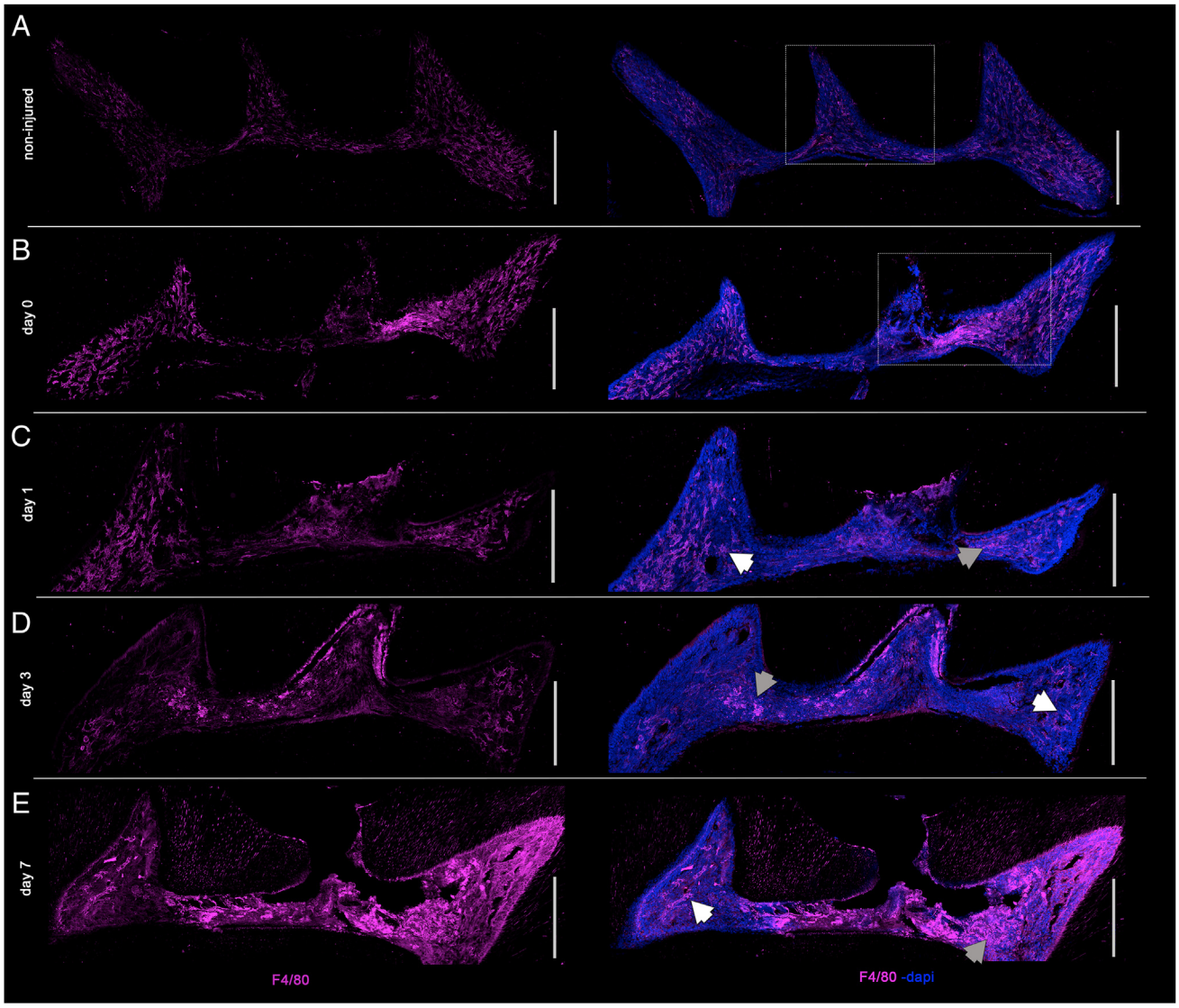

FIGURE 2 – Spatial and morphological changes of F4/80+ macrophages across different time points within the pulp chamber. *A*, Noninjured pulp. The *box* shows the intact middle pulp horn with densely and evenly located macrophages. *B*, Pulp, just right after the injury. The *box* shows the injured mid-pulp horn where macrophages are not visible and a part of the non-injured pulp horn where macrophages with their distinct morphology are present. *C*, *D*, and *E*, Pulp, one day, three days, and seven days after injury. The *white arrows* indicate the morphologically intact macrophages within the non-injured pulp horn. In contrast, the gray arrows point out an area where the morphology of the macrophages looks less distinct. The middle pulp horn is the mechanically injured pulp horn in each image. The first column includes images showing F4/80+ immunofluorescence, and the second column shows F4/80 co-localization with dapi. Scale bars are 200 μm. Magnification: 20X.

F4/80+ Macrophages are Resident and Respond to Pulp Injury

Histologically and kinetically, we studied macrophages in response to injury. On days 1, 3, and 7 after injury, the morphology of macrophages in noninjured areas of the pulp was similar to healthy pulp tissue with amoeboid cell shape with numerous cytoplasmic extensions present (Fig. 2*A*, *C*, and *D*). However, histologically, on day 3 after injury in the areas close to the injury site and on day 7 within the whole coronal pulp, macrophages exhibited an enlarged amoeboid shape with fewer cytoplasmic extensions (Fig. 2*D* and *E*). The timing and location of these morphological changes of macrophages corresponded well with the expansion timeline of the bacteria. We also quantitatively studied the changes in F4/80+ macrophages in response to injury, given that they were the main resident immune cell population at baseline. Out of total CD45+ immune cells, the total percentages of F4/80+ macrophages decreased at day 1 ($P = .001$) and 7 ($P = .001$) compared to a healthy pulp, which is likely due to the concomitant increased proportions of neutrophils and monocytes (Fig. 3*J*). However, by cell count, the total number of F4/80+ cells remained the same across all time points in pulp tissues (Fig. 3*I*). These findings indicate that macrophages are the main resident immune cells of the pulp tissue and respond to injury.

Cd11b + Myeloid Cells, Predominantly Neutrophils, Are the Major Immune Cell Populations up to Seven Days After Pulp Injury

Upon injury, compared to healthy pulp tissue, the percentage and total number of CD45+ immune cells increased, constituting about

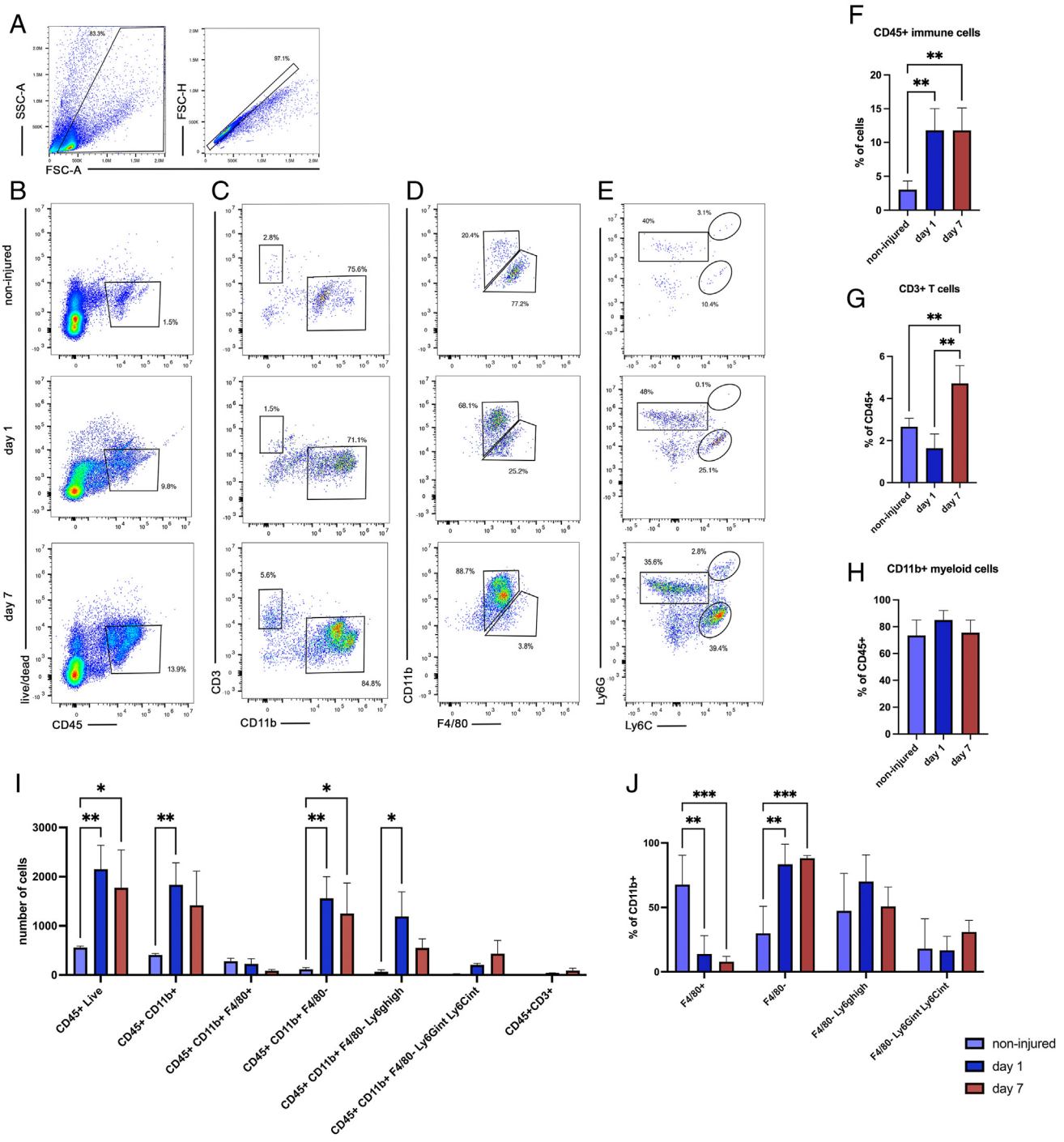

FIGURE 3 – Myeloid cells, especially neutrophils, are the major immune cell populations up to 7 days after pulp injury. *A*, Representative flow cytometry (FC) plots showing the initial steps of the gating strategy. *B*, Representative FC plots to gate CD45+ live immune cells of non-injured pulp, injured pulp one and seven days after injury. *C*, Representative FC plots to gate CD3+ T cells and CD11b + myeloid cells of non-injured pulp, injured pulp one and seven days after injury. *D*, Representative FC plots to gate F4/80+ macrophages of non-injured pulp, injured pulp one and seven days after injury. *E*, Representative FC plots to gate F4/80- myeloid cells into Ly6Ghigh, Ly6Gint Ly6Cint, and Ly6Ghigh Ly6Chigh neutrophils/monocytes of non-injured pulp, injured pulp one and seven days after injury. *F*, % of CD45+ immune cells among all cells at different time points. *G*, % of CD3+ T cells among CD45+ cells at different time points. *H*, % of CD11b + myeloid cells among CD45+ immune cells at different time points. *I*, Number of immune cell changes plotted from three independent experiments. *J*, % of different myeloid cell populations (macrophages, neutrophils, and monocytes) at different time points. *I*, Two-way ANOVA with Tukey's multiple comparisons test. (type of immune cells: $P < .0001$, F = 10.25, DOF = 6; time: $P < .0001$ F = 14.79, DOF = 2; interaction: $P = .07$, F = 1.7, DOF = 12). *J*, Two-way ANOVA with Dunnett's multiple comparisons tests (type of immune cells: $P < .0001$, F = 13, DOF = 3; time: $P = .7$ F = 0.3, DOF = 2; interaction: $P < .0001$, F = 7.9, DOF = 6). F, G, H-One-way ANOVA with Tukey's multiple comparisons test $^{*}P < .05$, $^{**}P < .01$, $^{****}P < .0001$. Mean ± SEM.

12% of all viable pulp tissue cells at day 1 and day 7 (Fig. 3*B* and *F*). The total number of CD45+ leukocytes also increased significantly on day 1 ($P = .001$) and on day 7 compared to baseline ($P = .015$) (Fig. 3*I*). Significant changes occurred within the myeloid immune cell population. Even though the percentage of CD11b + myeloid cells out of total immune cells remained similar on days 1 and 7, their numbers increased significantly compared to a healthy pulp (comparison of baseline to day 1; $P = .003$, baseline to day 7; $P = .051$) (Fig. 3*H* and *I*). The composition of this myeloid cell population also changed. Most of the myeloid cells in the healthy pulp tissue were F4/80+ macrophages. Upon injury, the major myeloid cell populations became CD11b + $Ly6G^{high}$ neutrophils and CD11b + $Ly6G^{int}Ly6C^{int}$ monocytes (Fig. 3*D*, *E*, and *J*). Among these neutrophil and monocyte populations, both the percentage and numbers increased significantly compared to healthy pulp and were similar on day 1 and day 7 after injury (comparison of percentages baseline to day 1; $P = .0001$, baseline to day 7; $P = 001$, comparison of numbers baseline to day; $P = .003$, baseline to day 7; $P = .025$) (Fig. 3*E*, *I*, and *J*). We also observed that CD3+ T cells significantly increased by day 7 post-injury from 2% at baseline to 4% of total immune cells (Fig. 3*G*).

DISCUSSION

To our knowledge, we have described for the first time the dynamic changes in immune responses up to day 7 in mice in the molar dental pulp injury model using both flow cytometry and histological analysis. We found that macrophages were the most abundant immune cell population in healthy molars, which aligns with a recent transcriptomics analysis of healthy mice and human molar teeth[6]. We also showed the spatial distribution of these macrophages by performing immunostaining. Their morphology and distribution in healthy pulp tissue resemble previous reports of antigen-presenting cells, including dendritic cells identified by antibodies in human tissue or mouse lines[20,21,27]. It is possible that these resident immune cells are F4/80+, the marker we used to identify resident macrophages, and cd11c+, which was the marker used to identify dendritic cell populations[28]. Another study also described CX3CR1+ macrophages with similar morphology histologically in the pulp tissue[27]. There is still limited understanding of how these resident macrophages behave during pulpitis and their primary role in orchestrating the immune response. Following injury, we found that F4/80+ macrophages adopted an amoeboid cell shape where cytoplasmic extensions were less visible. This change in morphology could be due to functional changes, including phagocytosis of bacteria or debris[29]. A recent study investigated the role of macrophages in dentin formation in a pulp capping model, finding that when clodronate liposome injections depleted macrophages, there was reduced dentin formation[30]. Further studies are needed to characterize the phenotype of macrophages, and whether their phenotype changes dynamically during pulpitis[22,30]. Also, further functional studies are essential to study macrophage behavior during pulpitis to understand how they drive innate immune response, including whether they play a role in bacterial clearance and recruitment of subsequent innate or adaptive immune cells.

In our study, the kinetics of neutrophil recruitment is interesting. Upon injury, we found neutrophils and monocytes were the primary immune cell populations recruited on day one. Neutrophils, in particular, were strongly recruited and remained as the prominent immune cell population up to day seven after injury. Ligature-induced periodontitis model study also showed that neutrophils expand on day one following inflammation however, both the percentage and the number of neutrophils decreased after day one[31]. Our findings of the continued presence of neutrophils in the pulpitis model may relate to the need to control bacterial invasion. A skin incision-wound healing model showed that after day two, there was a gradual decrease in the number of neutrophils at the wound site[32]. By contrast, when the incision site was inoculated with the bacterial pathogen *S. aureus*, neutrophils increased twofold compared to the vehicle group up to six days after bacterial inoculation[32]. This suggests that a consistent bacterial presence and release of pathogen-associated-molecular-patterns (PAMPS) may induce longer-lasting neutrophil recruitment to the infected site[9]. In our pulpitis model, we observed that bacteria presence was expanded where they penetrated deeper into the pulp tissue over time, which could have led to a more prolonged neutrophil presence. Characterization of the innate immune response in the pulpitis model further, by studying the response at an intermediate time point, such as day three, and later time points will allow a more comprehensive understanding of the macrophage and neutrophil dynamics, as well as the eventual transition to the adaptive immunity.

Our flow cytometry analysis showed that there was a continuity of acute inflammation and predominately innate immune responses on day seven after injury, with some evidence for a transition to adaptive immune responses and chronic inflammation. Immune profiling of later time points can provide more insight into the transition into adaptive immunity. There is also discussion on whether human dental pulp tissue has a lymphatic system and how it functions[33-35], raising questions about how the adaptive immune response might also differ in the pulp tissue compared to other tissues. We found that there was an increase in the T cell population on day 7. There is also evidence of T and B cell presence in inflamed pulp due to caries in studies investigating pulp tissue samples from human teeth[36]. Of note, the model we are using in mice expedites the process of a much slower disease progression in humans during pulpitis due to caries. Also, there could be species-specific differences in the role of innate and adaptive immune cells in pulpitis. Future studies will be needed to dissect the role of distinct innate and adaptive immune populations in pulpitis pathogenesis.

CONCLUSION

Macrophages are the primary immune cell population in healthy pulp tissue and their cellular morphology changes during pulpitis. Flow cytometry analysis showed rapid recruitment of neutrophils and monocytes during the early stages following injury, with the persistent presence of neutrophils throughout the analysis up to day 7. Histological analysis showed that while most of the bacteria were primarily localized at the injury site, they began to spread out within the pulp chamber by the endpoint. There is limited transitioning into adaptive immune responses after bacteria-induced pulpitis in mice molars during the initial seven-day period. Therefore, a dynamic innate immune response accompanies the bacterial invasion of the pulp in a mouse model of pulpitis.

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REFERENCES

1. Burns LE, Kim J, Wu Y, et al. Outcomes of primary root canal therapy: an updated systematic review of longitudinal clinical studies published between 2003 and 2020. Int Endod J 2022;55:714–31.
2. European Society of Endodontology, Duncan HF, Galler KM, Tomson PL, et al. European Society of Endodontology position statement: management of deep caries and the exposed pulp. Int Endod J 2019;52:923–34.
3. Taha NA, Khazali MA. Partial pulpotomy in mature permanent teeth with clinical signs indicative of irreversible pulpitis: a randomized clinical trial. J Endod 2017;43:1417–21.
4. Asgary S, Eghbal MJ, Ghoddusi J. Two-year results of vital pulp therapy in permanent molars with irreversible pulpitis: an ongoing multicenter randomized clinical trial. Clin Oral Investig 2014;18:635–41.
5. Gaudin A, Renard E, Hill M, et al. Phenotypic analysis of immunocompetent cells in healthy human dental pulp. J Endod 2015;41:621–7.
6. Krivanek J, Soldatov RA, Kastriti ME, et al. Dental cell type atlas reveals stem and differentiated cell types in mouse and human teeth. Nat Commun 2020;11:4816.
7. Galler KM, Weber M, Korkmaz Y, et al. Inflammatory Response Mechanisms of the Dentine-Pulp Complex and the Periapical Tissues. Int J Mol Sci 2021;22:1480.
8. Segal AW. How neutrophils kill microbes. Annu Rev Immunol 2005;23:197–223.
9. de Oliveira S, Rosowski EE, Huttenlocher A. Neutrophil migration in infection and wound repair: going forward in reverse. Nat Rev Immunol 2016;16:378–91.
10. Perez-Figueroa E, Alvarez-Carrasco P, Ortega E, Maldonado-Bernal C. Neutrophils: many ways to die. Front Immunol 2021;12:631821.
11. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. Nat Rev Immunol 2013;13:159–75.
12. Rosales C. Neutrophils at the crossroads of innate and adaptive immunity. J Leukoc Biol 2020;108:377–96.
13. Schultz BM, Acevedo OA, Kalergis AM, Bueno SM. Role of Extracellular Trap Release during Bacterial and Viral Infection. Front Microbiol 2022;13:798853.
14. Chamardani TM, Amiritavassoli S. Inhibition of NETosis for treatment purposes: friend or foe? Mol Cell Biochem 2022;477:673–88.
15. Nakamura K, Yamasaki M, Nishigaki N, et al. Effect of methotrexate-induced neutropenia on pulpal inflammation in rats. J Endod 2002;28:287–90.
16. Holder MJ, Wright HJ, Couve E, et al. Neutrophil extracellular traps exert potential cytotoxic and proinflammatory effects in the dental pulp. J Endod 2019;45:513–520.e513.
17. Duncan HF, Cooper PR. Pulp innate immune defense: translational opportunities. J Endod 2020;46(9S):S10–8.
18. Renard E, Gaudin A, Bienvenu G, et al. Immune cells and molecular networks in experimentally induced pulpitis. J Dent Res 2016;95:196–205.
19. Yoshiba N, Yoshiba K, Nakamura H, et al. Immunohistochemical localization of HLA-DR-positive cells in unerupted and erupted normal and carious human teeth. J Dent Res 1996;75:1585–9.
20. Sakurai K, Okiji T, Suda H. Co-Increase of nerve fibers and HLA-DR- and/or factor-XIIIa-expressing dendritic cells in dentinal caries-affected regions of the human dental pulp: an immunohistochemical study. J Dent Res 1999;78:1596–608.
21. Yoshiba K, Yoshiba N, Iwaku M. Class II antigen-presenting dendritic cell and nerve fiber responses to cavities, caries, or caries treatment in human teeth. J Dent Res 2003;82:422–7.
22. Yoshiba N, Edanami N, Ohkura N, et al. M2 phenotype macrophages colocalize with schwann cells in human dental pulp. J Dent Res 2020;99:329–38.

23. Lee C, Ramsey A, De Brito-Gariepy H, et al. Molecular, cellular and behavioral changes associated with pathological pain signaling occur after dental pulp injury. Mol Pain 2017;13:1744806917715173.

24. Gibbs JL, Urban R, Basbaum AI. Paradoxical surrogate markers of dental injury-induced pain in the mouse. Pain 2013;154:1358–67.

25. He Y, Gan Y, Lu J, et al. Pulpal tissue inflammatory reactions after experimental pulpal exposure in mice. J Endod 2017;43:90–5.

26. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. Nat Methods 2012;9:676–82.

27. Hong S, Lee J, Kim JM, et al. 3D cellular visualization of intact mouse tooth using optical clearing without decalcification. Int J Oral Sci 2019;11:25.

28. Cao Q, Wang Y, Wang XM, et al. Renal F4/80+ CD11c+ mononuclear phagocytes display phenotypic and functional characteristics of macrophages in health and in adriamycin nephropathy. J Am Soc Nephrol 2015;26:349–63.

29. Davies LC, Jenkins SJ, Allen JE, Taylor PR. Tissue-resident Macrophages. Nat Immunol 2013;14:986–95.

30. Neves VCM, Yianni V, Sharpe PT. Macrophage modulation of dental pulp stem cell activity during tertiary dentinogenesis. Sci Rep 2020;10:20216.

31. Alvarez C, Abdalla H, Sulliman S, et al. RvE1 impacts the gingival inflammatory infiltrate by inhibiting the T cell response in experimental periodontitis. Front Immunol 2021;12:664756.

32. Kim MH, Liu W, Borjesson DL, et al. Dynamics of neutrophil infiltration during cutaneous wound healing and infection using fluorescence imaging. J Invest Dermatol 2008;128:1812–20.

33. Bernick S. Lymphatic vessels of the human dental pulp. J Dent Res 1977;56:70–7.

34. Gerli R, Secciani I, Sozio F, et al. Absence of lymphatic vessels in human dental pulp: a morphological study. Eur J Oral Sci 2010;118:110–7.

35. Wisniewska K, Rybak Z, Szymonowicz M, et al. Review on the lymphatic vessels in the dental pulp. Biology 2021;10:1257.

36. Hahn CL, Falkler WA Jr, Siegel MA. A study of T and B cells in pulpal pathosis. J Endod 1989;15:20–6.