Mast Cells Contribute to the Immunomodulatory Effect of the Biomaterial Microenvironment in a Gender Specific Manner

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Abstract:

Regulation of the immune response contributes to the severity and outcomes in various disease conditions. Bioactive immunomodulatory biomaterials have shown promise for influencing these responses to promote tissue repair and regeneration. In this study, we investigated the role of mast cells in the regulation of the immune response to biomaterial scaffolds. In mast cell-deficient mice, there was dysregulation of the expected M1 to M2 macrophage transition typically induced by the biomaterial scaffold. Polarization progression deviated in a gender specific manner with an early transition to an M2 profile in female mice, while the male response was unable to properly transition past a pro-inflammatory M1 state. Both were reversed with mast cell adoptive transfer. Further investigation of the later stage immune response in male mice determined a sustained pro-inflammatory gene expression profile in deficient mice consisting of members from the IL-1 cytokine family and related downstream pathways. As mast cells were mainly associated with detrimental pro-inflammatory outcomes for biomaterial scaffolds, these results demonstrate their contribution to induced immunomodulatory therapies and support their potential as a critical immune regulatory element that can be manipulated for stimulating endogenous tissue repair.

Key Words: Immune Response, mast cell, decellularized extracellular matrix (ECM),

The immune response is known to have a necessary role in tissue homeostasis, remodeling, and repair, and our understanding of the involved immune populations and their phenotypic state improves our capacity to treat adverse disease conditions. Mast cells are tissue resident granulocytes classically associated in host defense, proinflammatory responses and various immune disorders[1]. The granules of mast cells are known to contain a variety of immune mediators demonstrated to contribute to pathological responses such as allergic reactions, inflammatory cell recruitment[2], and fibrosis[3]. While mast cells are also known to secrete a plethora of pro-remodeling/anti-inflammatory cytokines that are relevant for tissue repair, their role in tissue regenerative therapies has not been studied.

Biomaterial scaffolds that influence the immune response are emerging as a viable clinical strategy for tissue repair and regeneration[4]. Although this immunomodulatory influence associated with improved tissue outcomes has been rigorously evaluated with macrophage[5, 6] and T cell populations[7, 8], potential contribution of mast cells to the immunomodulatory effect of naturally derived biomaterials has been largely ignored. Studies have mainly just evaluated shifts in mast cell numbers associated with biomaterial deployment, though their general low numbers in tissue make it difficult to determine conclusive results from this approach[9, 10]. Early studies with synthetic materials have focused on their functional role and mainly produced results characteristic of a classical mast cell phenotype highlighting contributions to early acute inflammatory cell infiltration[2], and fibrous capsule formation[11]. From a general tissue disease perspective, several studies have similarly emphasized their detrimental role to tissue remodeling outcomes[12-14]. However, research has also produced confounding results[15-18] or evidence of their beneficial contributions to select cases of native tissue repair or inflammatory resolution[16, 19, 20], suggesting a greater functional complexity and potential to be utilized in therapeutic design. Based on recent evidence suggesting that mast cells can have beneficially influences on native tissue remodeling outcomes, we explored the role mast cells play in regulation of biomaterial scaffold-induced immunomodulatory responses, demonstrating its critical regulatory contribution to the pro-inflammatory to anti-inflammatory/pro-remodeling transition that deviates in a gender specific manner.

Given the recent clinical and rapidly expanding pre-clinical use of decellularized biomaterials derived from isolated tissue extracellular matrix (ECM), which are known to induce these effects based on immunomodulatory mechanisms[5, 7, 8],we chose to evaluate this class of biomaterial scaffold in $Kit^{W\text{-}sh/W\text{-}sh}$ mast cell-deficient mice. These mice are highly deficient in mast cells across various tissues and in the skin by 10-12 weeks while maintaining relatively normal levels of a broad range of lymphocyte and myeloid lineages[21], though some evidence of neutrophilia have been observed[22]. In particular, we used an injectable hydrogel form of myocardial matrix, which was previously shown to be immunomodulatory[23, 24] and recently progressed through a Phase I clinical trial in post-myocardial infarction patients[25]. To study the influence of mast cells on the response to biomaterial scaffolds, we subcutaneously injected the ECM hydrogel into the dorsal region of male and female $Kit^{W\text{-}sh}$ mast cell-deficient mice and compared the recruitment of surrounding immune cell populations to wild-type C57BL6/J mice. Day 1 isolated subcutaneous injections showed mast cell presence at low cellular density as early as day 1 post-injection. Mast cells were non-degranulated or minimally degranulated based on toluidine blue staining while deficient mice showed no mast cell presence in the material or throughout the skin (Fig. 1A, B). Similar characteristics of mast cells along with greater inward infiltration were observed for the ECM scaffold isolated at 3 days post-injection (Fig. S1A, B). Comparison with subcutaneous injection of milled urinary bladder matrix showed a similar non-degranulated state of mast cells bordering the material at day 3 post-injection demonstrating that these observations were not specific to the ECM hydrogel (Fig. S1C, D). Quantification of total cellular infiltration into the ECM hydrogel scaffold along with staining for Ly-6G/Ly-6C$^+$ neutrophil (Fig. 1C) and F4/80$^+$ macrophage populations (Fig. 1D) showed similar or greater infiltration in deficient mice compared to wild-type mice (Fig. 1E-J). These results

contrast with those observed with implanted synthetic materials where mast cell-deficiency led to decreased infiltration of macrophages and neutrophils[2], which mast cells are known to normally support through release of histamine, causing vasodilatation and increased vascular permeability, and chemokines. Furthermore, previously mentioned potential neutrophilia in this deficient model did not manifest in significant differences in this experimental context. *In vitro* assessment of the interaction of differentiated bone marrow derived FcεRI^{+}c-kit^{+} mast cells with the ECM hydrogel in solution at non-gelling concentrations in comparison to collagen and media only controls showed higher alamarBlue™ readings conveying matrix interaction enhanced mast cell viability (Fig. S2,3).

As ECM scaffold injection allowed for cellular infiltration similar to wild-type mice despite the lack of mast cells (Fig. 1), a screen of immune cell populations and polarized phenotypes through flow cytometry (Fig. S4-S5) and gene expression markers (Table S1) was utilized to assess if these infiltrating cells were affected by loss of regulatory contributions from mast cells. To confirm that this shift was mast cell specific, mast cell-engrafted mice were also similarly assessed where adoptive transfer of bone marrow derived mast cells differentiated *in vitro* were delivered subcutaneously into deficient mice to reconstitute the local resident mast cell population[21, 26]. Mast cell engraftment was confirmed by toluidine blue staining showing mast cells in dermal tissue and isolated ECM scaffold injections (Fig. S6). Transient polarization of immune cells from a pro-inflammatory to a pro-remodeling phenotype is a characteristic of wound healing responses[6, 27, 28] and the immunomodulatory effect of ECM scaffolds promotes this transition[8, 29]. Macrophage polarization assessment based on ratio of M2 pro-remodeling and M1 pro-inflammatory phenotypic markers in male mice showed a significant lack of polarization shift towards M2 for mast cell-deficient mice at day 11 post-injection that was observed in wild-type mice (Fig. 2A) and in previous studies[24]. These mice showed a restoration of the M2 shift, which was significant to the deficient group (Fig. 2A). Assessing the individual percentages of M2 and M1 macrophages showed similar percentages of M2 and M21 macrophages (Fig. S7C, G) for the wild-type and deficient mice while a higher relative number of M1 macrophage was present in deficient mice at the day 11 (Fig. S7E). Notably, addition of mast cells in the engrafted mice had a significantly greater percentage of M2 macrophages compared to deficient mice. This result further supports an alternative mast cell phenotype from ECM scaffold interaction as opposed to a classically activated phenotype that would promote greater M1 macrophage presence. In contrast, female mice showed an M2 dominant presence at the early day 3 timepoint (Fig. 2B), which arose from a higher relative percentage of M2 macrophages (Fig. S7D) and not from differences in M1 or M2 macrophage percentages (Fig. S7F, H). The early observation of a M2 dominant response could result from influences of female hormones such as estrogen, which has been demonstrated to accelerate resolution of pro-inflammatory responses[30]. Furthermore, female hormones are known to influence mast cell activation contributing to the enhanced host defense and greater incidents of autoimmune disease in females[1, 31]. Notably, there were no changes in either gender in total macrophage numbers (Fig. S7) and other assayed populations including T-cells subpopulations (Fig. S8), B cells (Fig. S9), dendritic cells (Fig. S10), and mast cells (Fig. S11). Thus, the results stemmed from phenotypic changes in immune cell polarization versus differences in general immune cell numbers between models (Fig. 1).

A gene expression screen of select pro-inflammatory and pro-remodeling/anti-inflammatory markers between the wild-type and deficient group showed only a significant increase in anti-inflammatory Il38 in deficient compared to wild-type at day 3 post-injection (Fig. S12A). At day 11 when significant shifts based on flow cytometry were observed, significant differences in the IL-1 family including increases in pro-inflammatory Il1b and Il33 expression, and a decrease in Il38 (Fig. S12B). Further comparison with engrafted mice showed that increased expression of Il1B relative to housekeeping gene, Gapdh, and IL-1 receptor antagonist, Il1rn, (Fig. 2C, F) and Il33 in deficient mice were reversed in the engrafted mice

supporting mast cell influence on the IL-1 cytokine family response (Fig. 2D). Notably, expression of Cd206 was significantly upregulated in engrafted mice compared to wild-type controls, matching previous flow cytometry results of an increased M2 macrophage transition from mast cell interaction with the ECM scaffold (Fig. 2E, Fig. S7C). Similar assessment for female mice at day 3 when dysregulation of macrophage polarization was observed by flow cytometry (Fig. 2B) determined only a significant upregulation of M1 marker Nos2 in deficient mice compared to wild-type that was reversed in engrafted mice (Fig. 2G). Though this result could be considered contradictory to the flow cytometry results suggesting a greater M2 profile at this timepoint (Fig. C,D), studies on the influence of female hormones have shown specific upregulated expression of Nos2[32, 33], potentially suggesting the known interaction of mast cells with female hormones helps to regulate these responses[34]. Overall, loss of mast cells in male mice inhibited pro-inflammatory to pro-remodeling transition of macrophage and cytokine profiles, while this transition was accelerated in female mice.

There are known limitations to summarizing the immune response with a few selected markers as a wide array of immune cell populations and cytokine mediators are known to influence the immune response. Thus, a more comprehensive multiplex analysis was carried out through a Nanostring panel directly probing the RNA molecular content for 561 of immune related targets. Day 11 male mouse samples were assessed as results supported a lack of pro-inflammatory resolution in deficient mice at this timepoint. PCA analysis showed that the majority of wild-type and engrafted samples clustered together. Deficient samples were mostly separately grouped and not tightly clustered conveying increased variance due to removal of regulatory influence from mast cells (Fig. S14). Similarly, Z-score heatmap of normalized gene expression showed a differing immune profile in deficient samples compared to wild-type and engrafted samples (Fig. S15). Differential expression analysis of the deficient compared to the wild-type and engrafted response determined differentially upregulated genes for a host of pro-inflammatory cytokines (Il1b, Il6, Ifng, Tnf) and chemokines (Ccl3, Ccl4, Ccl7, Cxcl1, Cxcl3) (Fig. 3, Table S2). Compensatory upregulation of anti-inflammatory markers such as Il10, Il19, and Cxcl10 was also observed, which commonly occur in cases of pathological tissue remodeling and foreign body responses[35, 36]. Pairwise analysis showed a similar pro-inflammatory differential response profile between deficient with either wild-type or engrafted expression. As expected, mast cell marker, Fcer1a, was decreased compared to mast cell containing models (Fig. S16, Table S3, S4). Notably, differential expression of genes between the engrafted and wild-type samples lacked several pro-inflammatory markers particularly associated with the IL1 cytokine family and mainly consisted of cellular processes, cell cycle, and migration markers (Fig. S17, Table S5). Gene enrichment analysis between the deficient versus the wild-type and engrafted response through KEGG pathway analysis highlighted several pro-inflammatory pathways such as TNF, IL-17, and NF-κB, and cellular stress pathways such a necroptosis and apoptosis (Fig. S18, Table S6). Enrichment analysis through the top 20 GO terms similarly supported lack of transition from a pro-inflammatory to pro-remodeling response in the deficient with enrichment of pathways related to neutrophils, inflammatory responses and pro-inflammatory cytokines such as IL-6 and IL-1 (Fig. 3B, Table S7, S8). In comparison, GO term analysis between the engrafted versus wildtype response lacked these enriched inflammatory pathways instead highlighting pathways related to general immune cell proliferation, activation, regulation, and adhesion. Ultimately, this comprehensive gene expression profiling demonstrated that mast cells play a role in the immunomodulatory transition from a pro-inflammatory to pro-remodeling response and support enhanced regulation of immune cellular processes for ECM based biomaterial scaffolds.

This study demonstrated that mast cells contribute to the dynamic immunomodulatory response to pro-regenerative biomaterial scaffolds. Therapeutics targeting regulatory immune cell populations such as T cells have allowed for the creation and advancement of various immuno- and biomaterial therapies. Our work suggests that attention and targeting to the

immunoregulatory effects of mast cells could translate to further synergistic coordination of tissue remodeling responses for improved tissue regeneration therapies.

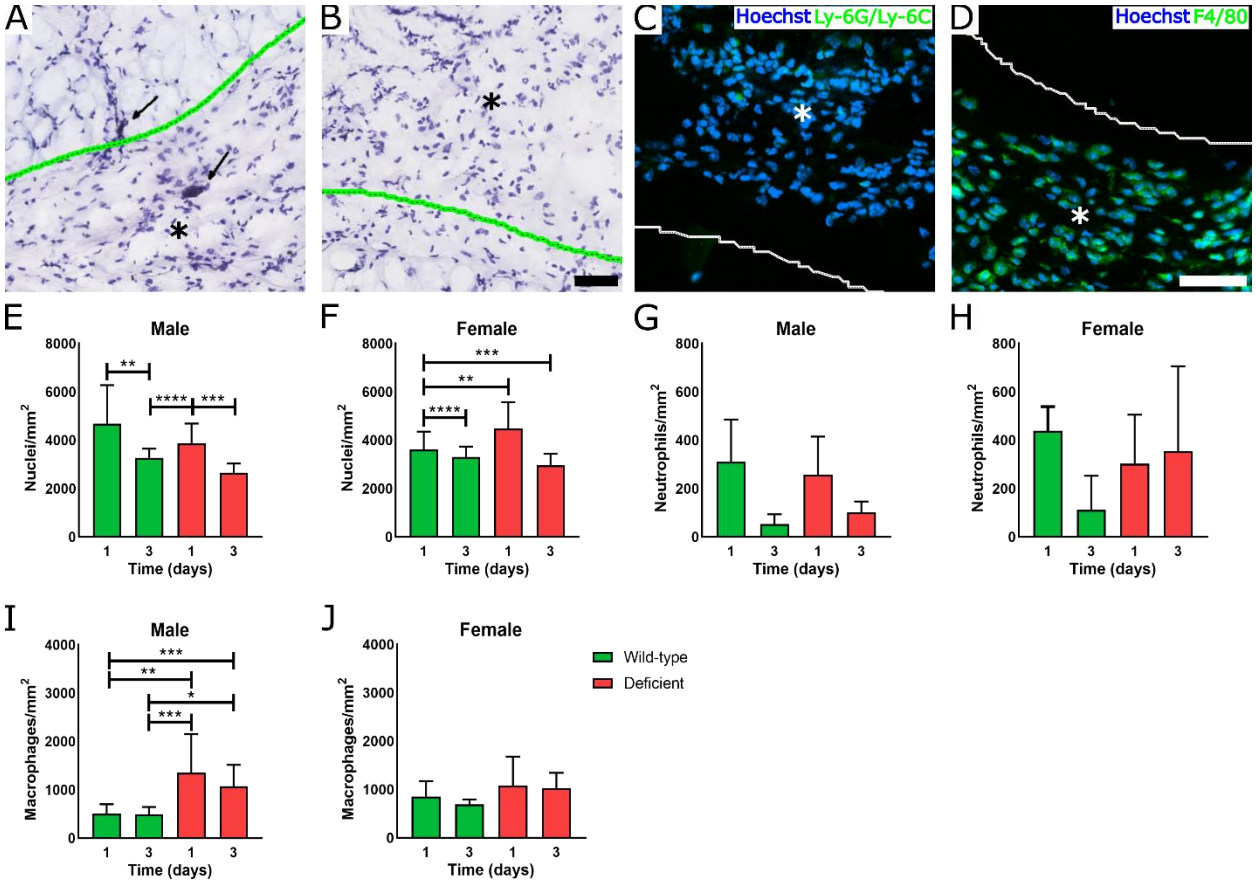

Figure 1: ECM scaffold promotes early immune cell infiltration with or without mast cells. Representative images of toluidine blue staining of ECM scaffold (green outlined with asterisk where material is present) and neighboring dermal tissue in wild-type (A) and mast cell-deficient (B) mice at day 1 post-injection. Mast cells can be found as early as day 1 showing lack of or minimal degranulation (black arrow). Representative fluorescent images of injected biomaterial (white outlined with asterisk) with Ly-6G/Ly-6C^{+} neutrophil (C) and F4/80^{+} pan-macrophage staining (D) in green, and Hoechst nuclei counterstain in blue. Quantification of total nuclei (E, F), neutrophil (G, H) and macrophage (I, J) density at one and three days post-injection for male and female wild-type (green) and deficient (red) mice. Scale bar of 50 µm in toluidine blue (A-B) and fluorescent images (C, D, G, H). (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$).

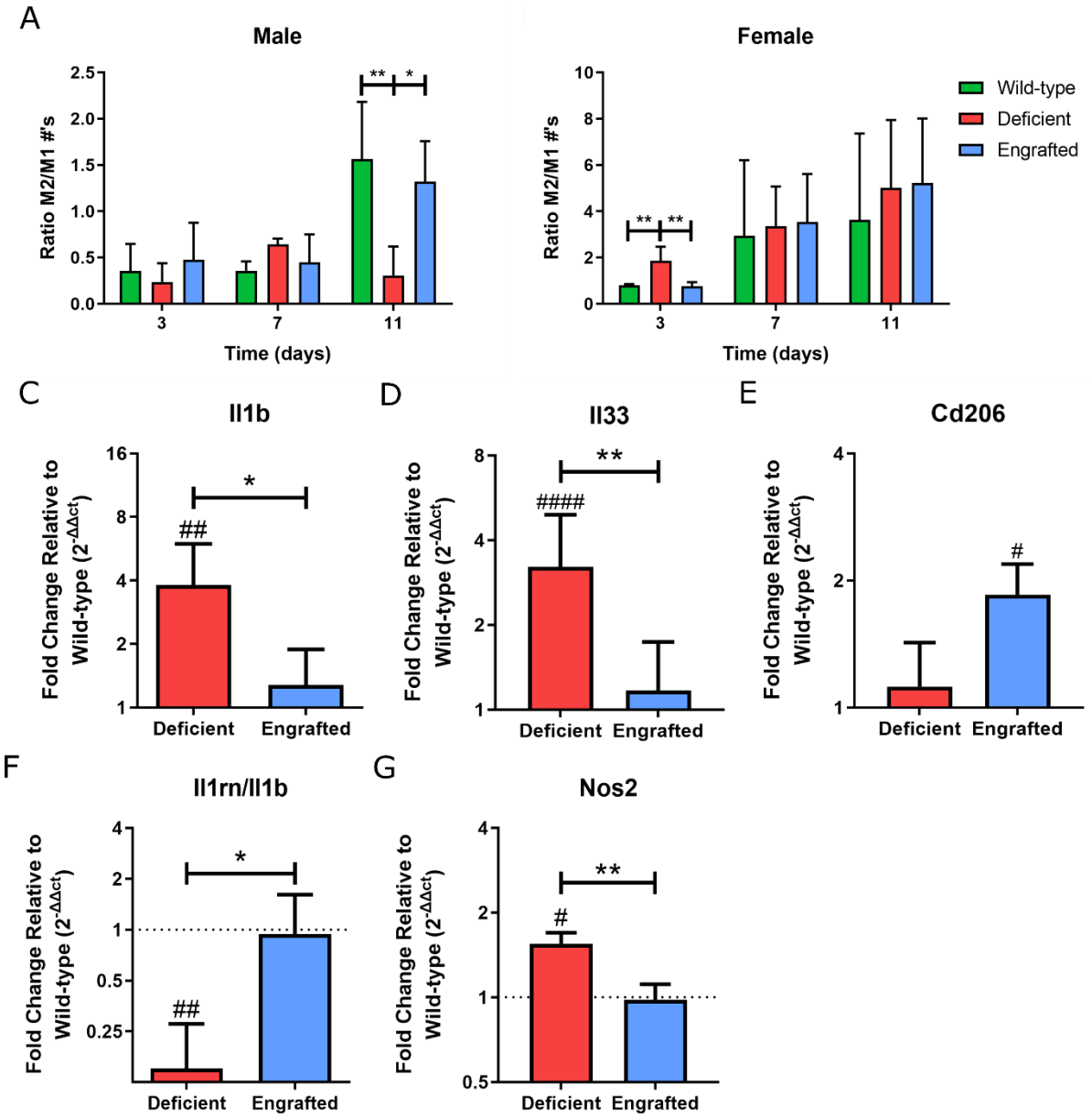

Figure 2: Transition from a pro-inflammatory to pro-remodeling macrophage and cytokine immune profile is dysregulated in a gender specific manner. Flow cytometry quantification for macrophages polarization based on a ratio of CD206$^+$ M2 versus CD86$^+$ M1 macrophage counts was assessed at three, seven, and eleven days post-injection between wild-type (green), mast cell-deficient (red), and mast cell-engrafted (blue) male (A) and female mice (B). Relative gene expression normalized to wild-type expression at day 11 in male mice measured by qPCR for (C) Il1b, (D) Il33, and (H) Cd206 relative to housekeeping gene, Gapdh. (F) Gene expression ratio of Il1b to receptor antagonist, Il1rn. (G) Relative gene expression at day 3 in female mice measured by qPCR for Nos2 relative GAPDH housekeeping gene. # indicates significance relative to wild-type expression, * conveys significance between deficient and engrafted, and gene expression plotted as mean ± SEM. ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#\#}p < 0.0001$, $^{*}p < 0.05$, and $^{**}p < 0.01$).

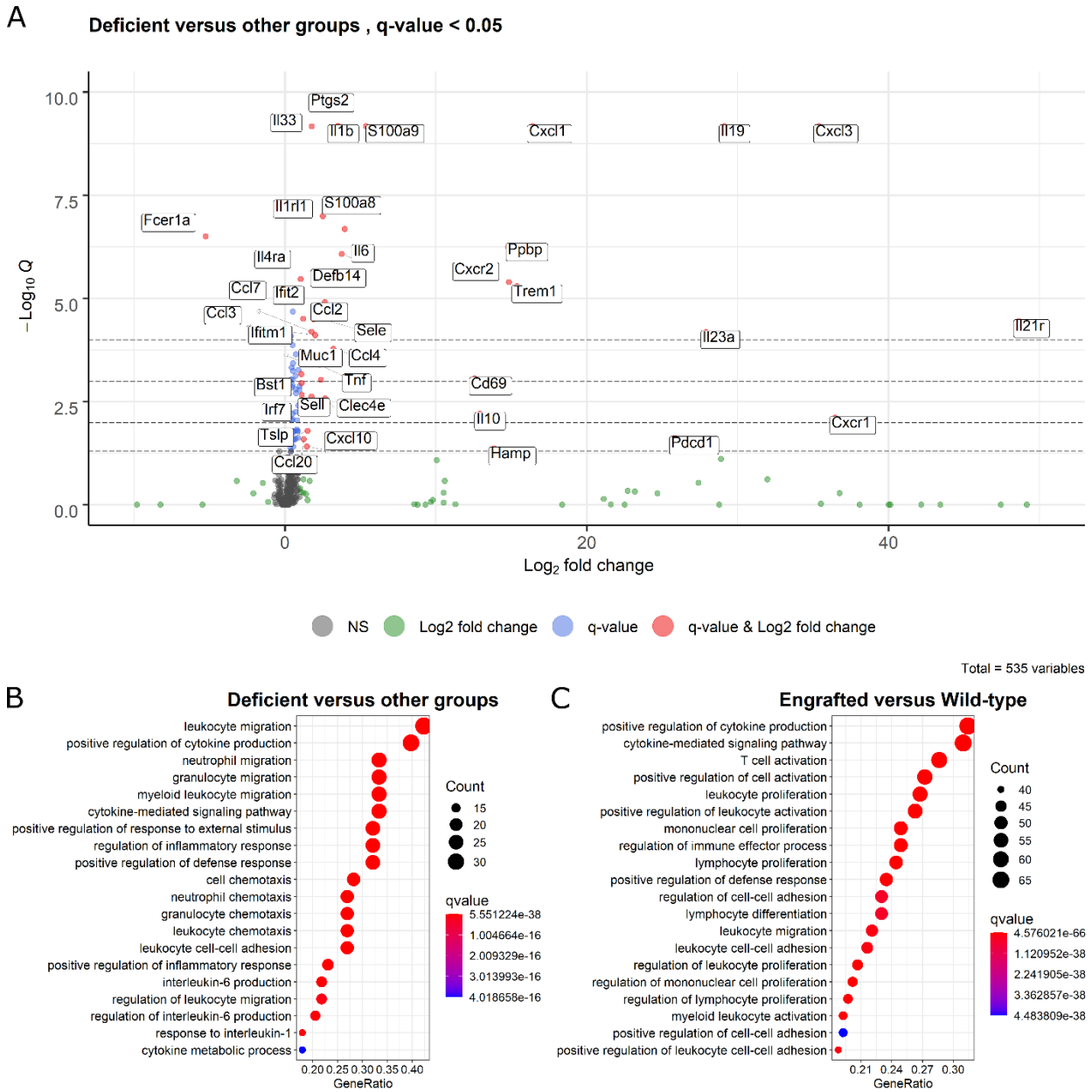

Figure 3: Mast cell-deficient male mice maintains late pro-inflammatory response to biomaterial implantation while mast cell-engrafted mice restores similar immunomodulatory effects. (A) Differential expression with dots indicating false discovery rate less than 0.05 (blue, red), absolute fold change greater than 1.5 (green, red) or non-significant (black). Dashed lines indicating different significance thresholds (**q* < 0.05, ***q* < 0.01, ****q* < 0.001 and *****q* < 0.0001). Dot plot of top 20 biological process GO terms based on differential expressed genes with a false discovery rate of q-value < 0.05 between the (B) deficient versus wild-type and engrafted groups, and (C) the engrafted versus the wild-type group. GO terms were sorted by gene ratio with size representing total counts and color representing q-value.

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