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Regulatory T Cells Orchestrate Similar Immune Evasion of Fetuses and Tumors in Mice

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Embryos and tumors are both masses of dividing cells expressing foreign Ags, but they are not rejected by the immune system. We hypothesized that similar tolerogenic mechanisms prevent their rejection. Global comparison of fetal and tumor microenvironments through transcriptomics in mice revealed strikingly similar and dramatic decreases in expression of numerous immune-related pathways, including Ag presentation and T cell signaling. Unsupervised analyses highlighted the parallel kinetics and similarities of immune signature downregulation, from the very first days after tumor or embryo implantation. Besides upregulated signatures related to cell proliferation, the only significant signatures shared by the two conditions across all biological processes and all time points studied were downmodulated immune response signatures. Regulatory T cell depletion completely reverses this immune downmodulation to an immune upregulation that leads to fetal or tumor immune rejection. We propose that evolutionarily selected mechanisms that protect mammalian fetuses from immune attack are hijacked to license tumor development. *The Journal of Immunology*, 2016, 196: 678–690.

There are striking similarities between fetal development and tumor development. They both rely on intense cell division, invasion of host tissues, and sustained vascularization (reviewed in Refs. 1, 2). In the physiological setting of pregnancy, trophoblast cells proliferate intensely, invade the maternal endometrial decidua, and then migrate into the uterine wall where they contribute to the blood supply. This is reminiscent of tumor development and led to the description of the trophoblast as a pseudo-malignant tissue (3, 4), or even a physiological metastasis (5).

Moreover, despite the fact that fetus and tumor express foreign Ags—paternal alloantigens for fetuses and altered autoantigens for tumors (6–8)—they are not rejected by the immune system. Numerous immune cells such as NK cells, regulatory T cells (Tregs),

effector T cells (Teffs), and dendritic cells (DCs) populate the maternal–fetal interface (9). Some of them, such as uterine NK cells, have a preponderant trophic function (10), whereas others have been shown to play an often redundant role in tolerance of the fetus. However, the interaction between these cells is poorly understood and there is as yet no integrated view of how the immune system is kept under control so as to prevent the elimination of the allogeneic fetus. Similarly, numerous innate and adaptive immune cells appear to participate in the tolerant environment that protects Ag-expressing tumor cells from immune destruction.

Although there are discrete similarities between tolerance to fetuses and to tumors that have intrigued scientists for decades (11), there was until recently no robust evidence of common mechanisms at work in the two settings. The discovery of Tregs, which are key players in immune tolerance, has uncovered their seemingly similar role in the two processes (12). In human pregnancy, Tregs are enriched at the maternal–fetal interface throughout healthy pregnancy (13, 14), whereas a decreased Treg pool is observed in recurrent miscarriage cases (15) and preeclampsia (16). In addition to Tregs, effector T cells may also influence the immune acceptance of the fetus. Some studies have reported the regulation of the effector immune response toward a predominant and favorable Th2 type rather than Th1 type immunity during pregnancy (17, 18). In human cancer, the situation is clearer, with the extent of tumor infiltration being most often associated with poor survival and increased infiltration of activated Teffs being favorable (19, 20).

In mice, tumor emergence as well as embryo implantation elicits a strikingly similar brisk Treg response (21). Tregs specific for self-antigens are recruited in tumor or uterine draining lymph nodes before Teffs are recruited, with the functional relevance of this being supported by the fact that Treg depletion leads to fetal (22, 23) or tumor (24) immune rejection. These observations led us to hypothesize that tumors hijack mechanisms of tolerance initially selected during evolution of the mammalian immune system to protect fetuses (25).

To substantiate this hypothesis, we aimed more broadly to compare immune tolerance mechanisms in the two settings. The immune system comprises large sets of diverse, circulating, interconnected cells, which cooperate through numerous signaling molecules and soluble factors. To capture this complexity and better understand the

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D.N.-B. performed all the experiments, analyzed the results, and participated in writing the manuscript; T.C., L.F., and M.G.R. participated in some experiments and N.D. in data analysis; and D.K. designed and supervised the study, analyzed the data, and wrote the first manuscript, which was reviewed by all authors.

The online version of this article contains supplemental material.

Abbreviations used in this article: DC, dendritic cell; DEG, differentially expressed gene; E, embryonic day; FDR, false discovery rate; GO, gene ontology; GSEA, gene set enrichment analysis; ICA, independent component analysis; IPA, Ingenuity Pathway Analysis; Teff, effector T cell; Treg, regulatory T cell.

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global nature of immune responses at work in the tumor microenvironment and uterine tissues, systems approaches are needed. We thus investigated fetal and tumor microenvironments using whole transcriptome microarray analysis, which is now well codified and standardized and for which there are numerous mature tools for experimental design, data analysis, and graphical visualization (26–29).

We show that Tregs are not only important players, but are the conductors of the immune orchestra that plays a similar score leading to tolerance to fetuses and to tumors.

Materials and Methods

Animals

BALB/c and C57BL/6 female mice, 6–8 wk of age, were from Elevage Janvier (Le Genest St. Isle, France). All mice were treated in accordance with European Union guidelines for animal experimentation.

Tumor experiments

B16F10 melanoma cells were obtained from the American Type Culture Collection. Cells (5×10^5) were injected s.c. into the right flank of C57BL/6 mice. Mice were sacrificed at 4 and 14 d after tumor inoculation. Tumor microenvironments were collected from punch biopsies always of the same size (2.5 mm diameter) centered on the inoculation site, thus collecting both tumor and surrounding tissue. Samples were incubated overnight in RNeasy lysis buffer (Qiagen) at 4°C and then transferred to –80°C for storage.

Maternal–fetal experiments

Embryonic day (E)4 to E12 allogestant C57BL/6 mice were from Elevage Janvier. The entire uterus (with fetal tissues) was collected and samples were incubated overnight in RNeasy lysis buffer (Qiagen) at 4°C and then transferred to –80°C for storage.

Sample size

Sample sizes were as follows: 1) for maternal–fetal experiments, uteri from nonpregnant mice (control samples), $n = 6$; E4, $n = 4$; E6, $n = 6$; E8, $n = 4$; E10, $n = 4$; E11, $n = 4$; and E12, $n = 5$; 2) for Treg depletion experiments, nonpregnant mice, $n = 5$; E12, $n = 5$; and PC61-treated mice at E12, $n = 4$; and 3) for tumor experiments, normal skin samples (control samples), $n = 7$; tumor-bearing mice at day 4 after tumor inoculation, $n = 4$; and at day 14 after tumor inoculation, $n = 5$.

Gene expression analyses

Samples from uterine tissues and tumor microenvironment were lysed and homogenized using a tissue lyser (Qiagen), and total RNA was purified using TRIzol (Invitrogen) according to the manufacturers' instructions.

RNA yield was assessed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). RNA integrity was assessed using an Agilent Bioanalyzer showing a quality RNA integrity number of 8–10 (Agilent Technologies). The RNA was processed using the Illumina TotalPrep RNA amplification kit protocol according to the manufacturer's protocol. Briefly, labeled complementary RNAs were hybridized overnight to Illumina MouseWG-6 v2.0 Expression BeadChip arrays. The arrays were then washed, blocked, stained, and scanned on an Illumina BeadStation following the manufacturer's protocols. Illumina BeadStudio software was used to generate signal intensity values from the scans. Genes were filtered out from the analysis when their expression was below the detection limit ($p < 0.05$) in at least two of three samples in both microenvironment and control groups. Next, data were normalized according to the quantile method using the limma R package and then log transformed.

The limma package was used to identify differentially expressed genes (Benjamini–Hochberg corrected $p < 0.05$) at days 4 and 14 (tumor microenvironment) or E4, E6, E8, E10, and E12 (maternal–fetal tissues). The limma package is freely available at: <http://www.bioconductor.org/packages/release/bioc/html/limma.html>.

Dataset quality assessment

Hierarchical clustering was performed using Euclidean distance and the Ward agglomeration method. Principal variance component analysis was performed using R version 3.1.3, which is freely available at: <http://www.bioconductor.org/packages/release/bioc/html/pvca.html>.

All datasets were deposited in the Gene Expression Omnibus repository (reference no. GSE68454): <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE68454>.

Identification of specific molecular signatures

Specific molecular signatures were generated and statistically tested using the independent component analysis (ICA) → gene set enrichment analysis (GSEA) method developed in Pham et al. (30).

Independent component analysis

The CRAN package fastICA (31) was used to carry out the ICA.

GSEA

Probe sets were formatted into the GMT file, a GSEA input file format. Probes were sorted following the score of the statistical test and used as a preranked list of RNK files. We used the weighted scoring scheme to compute the enrichment score. A false discovery rate (FDR) q value, indicating the probability of a false-positive score, was computed from the FDR and normalized enrichment scores were calculated using the data-bases of signatures.

A detailed explanation of GSEA can be found in Subramanian et al. (32).

Generation of functional modules of molecular signatures

Overlaps between significantly enriched signatures from GSEA could be visualized in Cytoscape (33) (version 2.8.3) with the Enrichment Map plugin (34). The Enrichment Map produced networks whose nodes represent signatures and whose edges represent mutual overlap. This approach groups highly redundant signatures together as modules. Only gene sets with an FDR p value of at least <0.05 were selected, and the mutual overlap coefficient between signatures was set at 0.8. The Enrichment Map was used to identify the biological processes discriminating pregnant mice (E4–E12) from nonpregnant mice as controls (and tumor-injected groups from the control group).

Modules of functionally related signatures were manually circled and assigned a label. The functional network was manually curated to remove modules containing fewer than three signatures, resulting in a simplified network map, as shown in Figs. 2, 4, and 5.

Similarity measure

To compare tumor microenvironment and uterine tissue–extracted signatures, we used Jaccard and GOSemSim similarity indexes. The Jaccard index (Ji) is the ratio between the intersection of two sets and its union (35). $J(A,B) = [\text{size of } (A \cap B)] / [\text{size of } (A \cup B)]$.

The GOSemSim index is described in Yu et al. (36) and was released under the GNU General Public License in the Bioconductor Project and is freely available at: <http://bioconductor.org/packages/2.6/bioc/html/GOSemSim.html>.

In vivo depletion of CD4⁺CD25⁺ T cells

Treg in vivo depletion was performed by i.p. injection of 500 μ g of an anti-CD25 mAb (PC-61.5.3 from Bio X Cell, West Lebanon, NH) at ~ 2.5 d postcoitum. This induces a $>80\%$ transient depletion of CD25^{high} cells for ~ 3 wk in lymph nodes of normal mice. Uterine environments of rejected fetuses from these mice were then analyzed.

Results

Dynamic downregulation of immune pathways revealed by supervised transcriptome analyses of the pregnant uterus

We first analyzed global changes in the RNA expression profile of uterine tissues induced by pregnancy. We generated a set of transcriptomic data from the uterus of nonpregnant mice and from mice at 4–12 d postfertilization, that is, the E4–E12 stages of pregnancy. We verified the quality of our transcriptome dataset using principal variance component analysis, which estimates within a dataset sources of variability due to biological or technical effects such as RNA concentration, RNA quality, date, position in the array, or experimental groups. We observed that the different experimental groups (i.e., the time for a given condition) accounted for $>70\%$ of the total variability, indicating that our dataset is of high quality (Supplemental Fig. 1A). Principal component analysis of normalized gene expression datasets revealed a clear separation between mice from the nonpregnant and early (E6, E8) or late pregnancy (E10, E11, and E12) groups (Supplemental Fig. 1B).

We first performed supervised analyses looking at differentially expressed genes (DEGs) in uterine tissues. At E4, we could already

detect >2000 genes that are highly significantly up- or downregulated (FDR q value < 0.01), as compared with nonpregnant mice (Fig. 1A). This number rapidly increased with time to >6000 regulated genes at

E10 and E12. These observations highlight that the pregnancy-induced gene expression changes in uterine tissues are rapid and extremely complex and cannot be reduced to one or a few pathways.

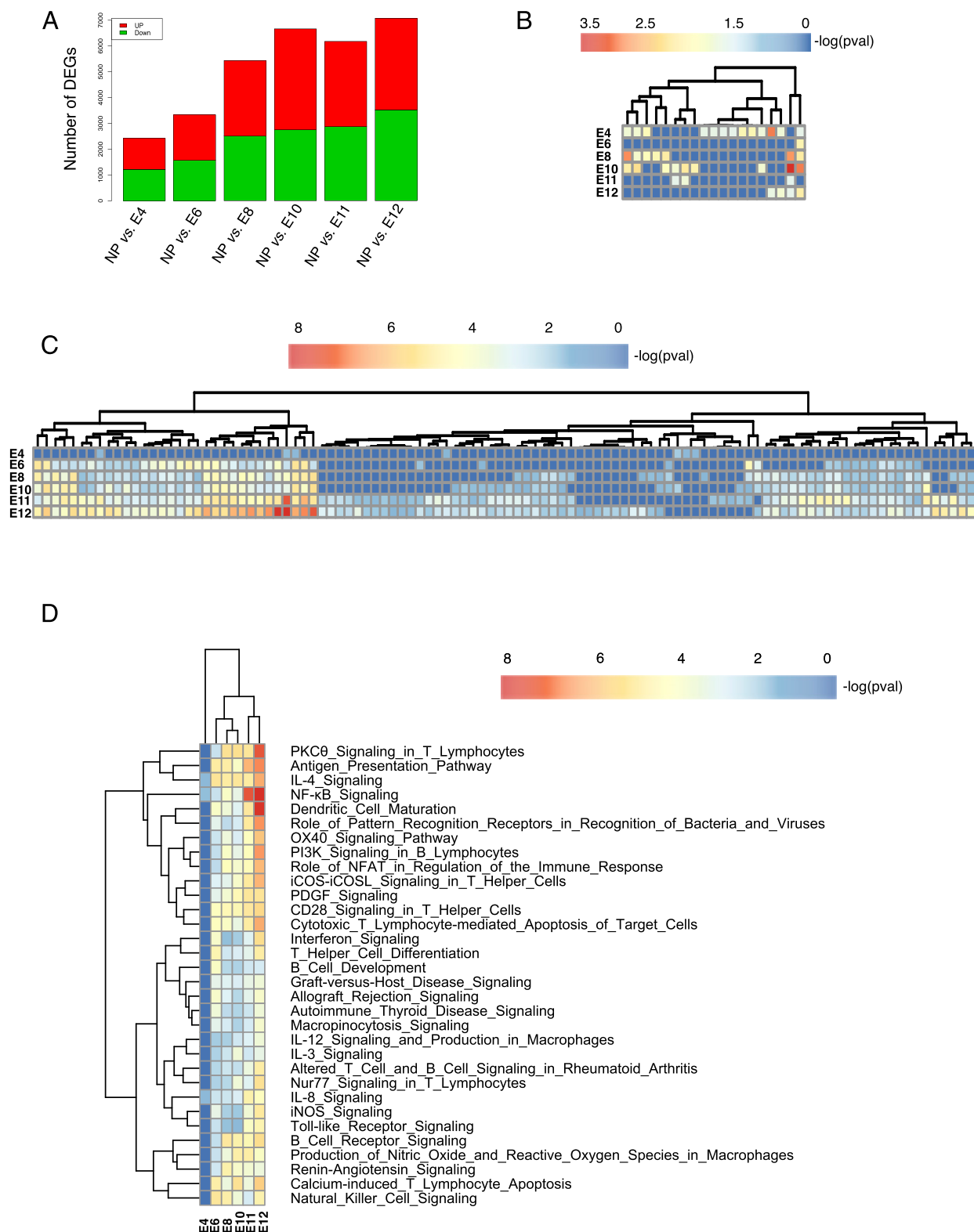


FIGURE 1. Supervised analyses of differentially expressed genes in uterine tissues during pregnancy. **(A)** Bar plot of all DEGs in pregnant mice at E4, E6, E8, E10, E11, and E12 in comparison with nonpregnant (NP) mice. The significance threshold was set at $p < 0.01$ (eBayes test). Shown in red are the upregulated genes and in green are the downregulated ones. **(B–D)** DEGs were annotated using IPA and depicted as heat maps according to their significance level [$-\log(p$ value)]. The upregulated immune-associated pathways are represented in (B) whereas downregulated ones are shown in (C) and (D). The heat map colors represent the statistical significance [$-\log(p$ value)] of each pathway. Only the pathways showing a $-\log(p$ value) > 1.3 are considered to be significantly modulated, which corresponds to a pale blue color as shown on the scale. Data are representative of $n = 4$ –6 mice per group.

To study the regulated genes participating in immune responses during pregnancy, we annotated DEGs using Ingenuity Pathway Analysis (IPA) and analyzed them as clusters of regulated genes participating in defined immune pathways. We identified 126 immune-annotated pathways as being significantly modulated at least at one time point. Only 19 (15%) of them were upregulated (Fig. 1B). Moreover, most of these pathways were not consistently upregulated over time, except for the antiproliferative role of TOB in T cell signaling pathway (Fig. 1B, *last column*). TOB being a negative regulator of T cell proliferation (37), its upregulation should in fact lead to decreased T cell responses.

In contrast, 106 pathways (85%) were consistently downregulated over time (Fig. 1C). Downregulated pathways were subdivided into two main subclusters based on statistical significance. The first cluster contains 32 highly significantly regulated pathways ($4 < -\log(p \text{ value}) < 8$) that were all downregulated already at E6 (Fig. 1D). Of note, 25 of these immune pathways (78%) are labeled signaling. They include the IL-4, IL-12, IFN, NF- κ B, and NFAT signaling pathways, which are all key to Th cell responses. The seven other downmodulated pathways are related to Ag presentation or T lymphocytes. The second cluster contains 47 pathways that are statistically less significant ($1.5 < -\log(p \text{ value}) < 3.5$) and are modulated later, starting at E10/E12 in most cases (Supplemental Fig. 1C). They include the IL-1, IL-17, CTLA4, CD40, and 4-1BB signaling pathways.

Dynamic downregulation of immune pathways revealed by unsupervised transcriptome analyses of the pregnant uterus

Supervised analyses may bias the interpretation of the results. Therefore, we further analyzed our datasets with unsupervised methodologies. We first used ICA, which blindly unravels the complexity of a dataset by identifying sources of complexity, whose variations of expression are correlated and define molecular signatures. In addition to these signatures generated from the dataset, we also supplemented our analysis with gene sets compiled from Gene Ontology (GO) (38) using GSEA (32). Altogether, the analysis identified 585–817 significantly enriched signatures from E4 to E12, and it focused on the highly discriminating ones, that is, with a Benjamini–Hochberg FDR of < 0.05 . Using Enrichment Map (34), a Cytoscape software (33) plugin, we represented them as networks of signatures, in green and red for the down and upregulated ones, respectively (Fig. 2A).

As early as E4, large groups of signatures categorized as related to 1) DNA and RNA synthesis, 2) ribosome, proteasome, or endoplasmic reticulum function, and 3) metabolism were upregulated. They likely reflect the intense proliferation of fetal and uterine cells. Of note, the only other signatures regulated at E4 were those related to the immune response, and they were all downregulated. The early trend for cell proliferation upregulation and immune response downregulation is then conserved from E4 to E12, with more numerous immune annotated signatures being downregulated over time (Fig. 2A).

Dynamic upregulation of Treg-related pathways in the pregnant uterus

Reductionist approaches have highlighted the role of Tregs in maternal–fetal tolerance (25). Therefore, we asked what the behavior of Treg signatures could be within our dataset. We found that a Treg signature defined by genes that are upregulated by Foxp3 were upregulated as early as E4 and remained so until E12 (Fig. 2B). In contrast, a mirror signature defining Tregs by genes that are downregulated by Foxp3 (39) was downmodulated (Supplemental Fig. 2A). Furthermore, unbiased principal component analysis, based on other described Treg gene expression

signatures (40–42), showed a perfect separation of pregnant and nonpregnant groups as early as E6 (Supplemental Fig. 2B–D). Altogether, these results are in line with the early recruitment and activation of Tregs in the pregnant uterus (25), and they indicate that Foxp3 does act as a transcriptional activator and repressor *in vivo*.

Ag presentation and T cell activation are the main downmodulated pathways in the pregnant uterus

We then analyzed the main significantly enriched immune pathways represented in our unsupervised signatures using IPA representation (Fig. 3, Supplemental Figs. 3, 4B). The first pathway, the Ag presentation pathway, shows early (E6) downmodulation (green) of MHC molecules and genes involved in proteolysis, transport, and presentation of peptides by MHC molecules (Fig. 3, *upper panel*), which is maintained and even more pronounced at E12. Altogether, this should result in a marked decrease of Ag presentation to T cells. A larger pathway called DC maturation reveals numerous additional downmodulated genes (Supplemental Fig. 4B). These include genes encoding membrane molecules involved in DC maturation 1) by T cells (MHC class I and class II for signal 1, and CD83, CD86, and CD40 for signal 2), 2) by innate lymphocytes (TNFR, CD40, and CD1A), or 3) by microbes and cytokines (TLR2/3/4/9).

To confirm further the downmodulation of Ag presentation–related gene expression and validate the data obtained by gene expression annotation, we tested whether transcriptional changes were reflected in changes of protein expression at the cell level. Therefore, we isolated DCs from nonpregnant or pregnant uteri and analyzed their numbers and phenotype. We observed a decrease in the percentages of DCs among hematopoietic cells in pregnant uteri, which could account for part of the decreased RNA expression. Moreover, we found reduced expression of activation markers such as MHC class II or TLR2 molecules (Supplemental Fig. 4A).

The second pathway, called CD28 signaling in Th cells, shows the decreased expression of MHC and costimulatory molecules on APCs, as discussed above, together with downregulation of multiple T cell activation pathways, including NF- κ B and NFAT. These early changes (E6) were also observed at E12.

Reversion from a down- to an upregulated immune microenvironment in the uterus by Treg ablation

To investigate whether changes in the maternal–fetal interface were controlled by Tregs, we mated female mice that were Treg-depleted and analyzed uterine tissues at E12 (Fig. 4). Comparison of unmanipulated pregnant and nonpregnant mice showed again major downregulation of immune response–annotated signatures and upregulation of cell proliferation–related signatures (Fig. 4A). In contrast, in Treg-depleted pregnant mice, the very same immune signatures were markedly upregulated in the uterine tissues of the rejected fetuses, compared not only with control pregnant mice (Fig. 4C) but also with nonpregnant mice (Fig. 4B). This indicates true immune activation and not just the reversion to baseline of the immune signature downmodulation observed in pregnant mice. Taken together, these results confirm that Tregs play an essential role in orchestrating tolerance in uterine tissues during pregnancy.

Remarkably similar dynamic changes in gene expression profiles in the microenvironment of tumors and fetuses

We previously showed that embryo or tumor cell implantation leads to a similar brisk recruitment of self-specific activated/memory Tregs (21, 25). To analyze the similarities between tolerance to

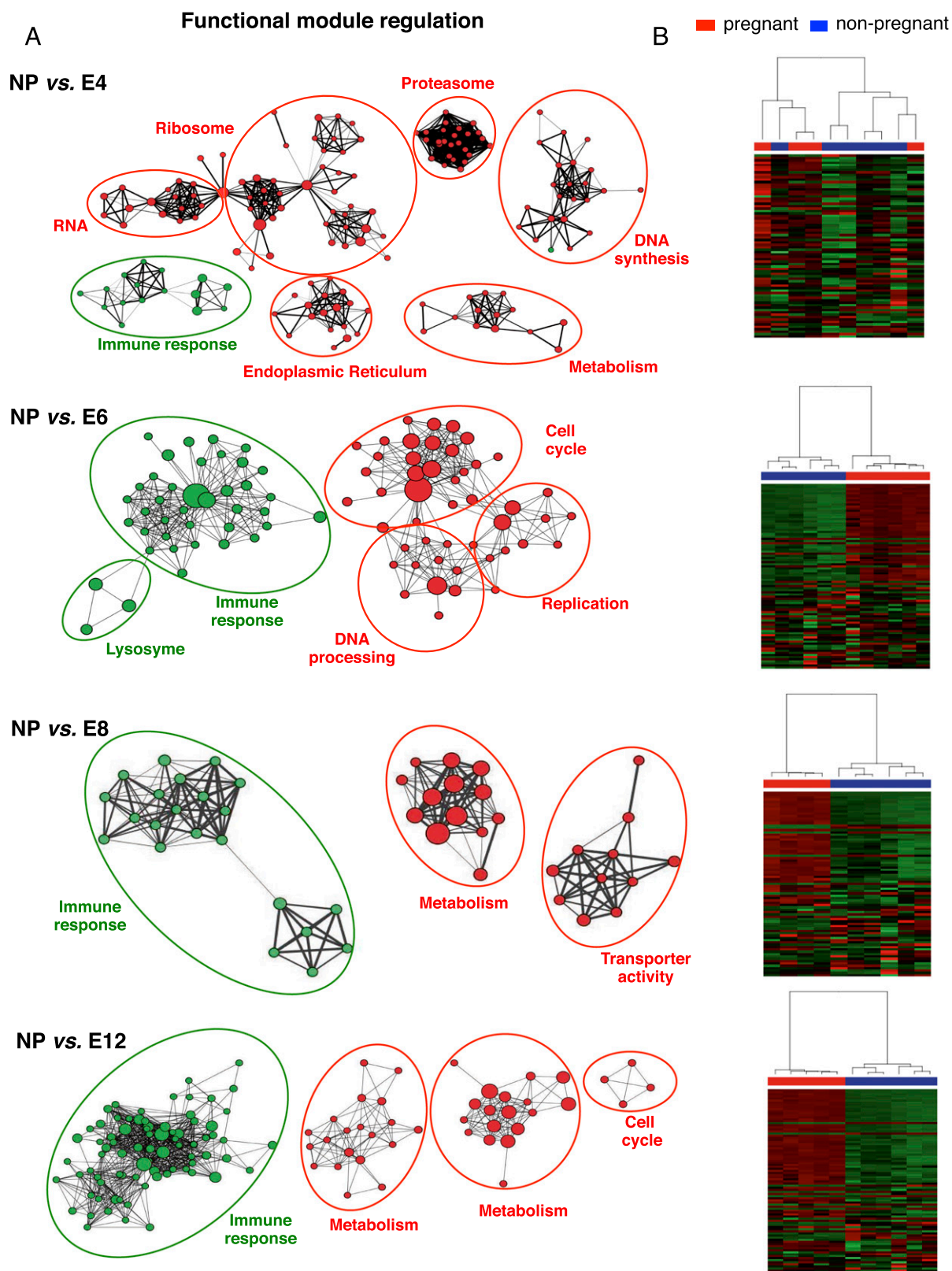
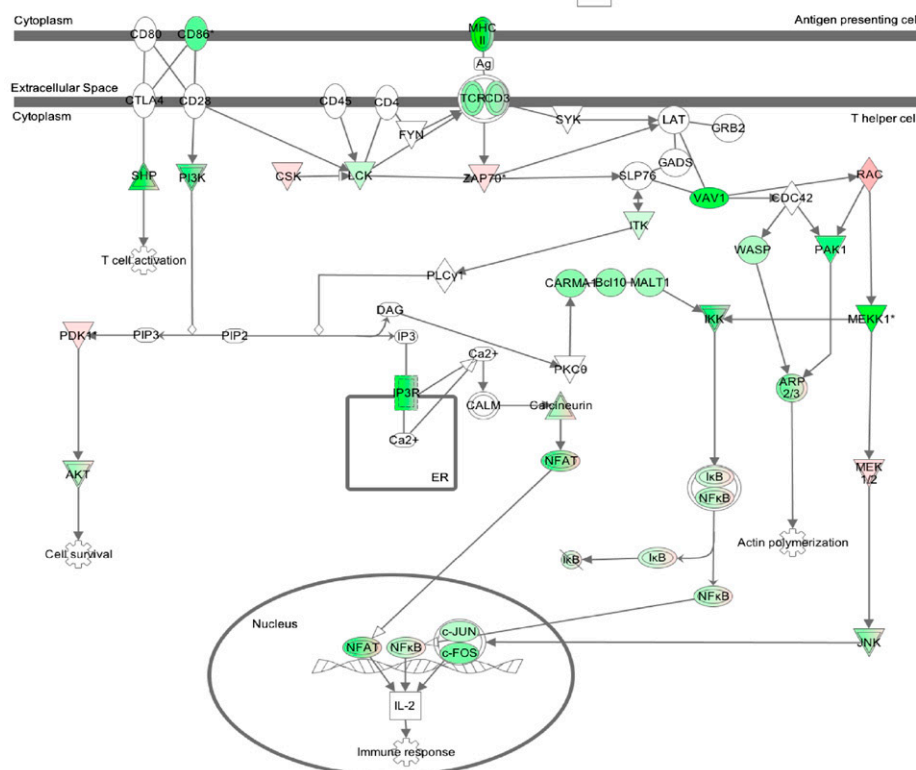


FIGURE 2. Unsupervised analyses of the dynamic regulation of pathways in uterine tissues during pregnancy. **(A)** Cytoscape representation. The enrichments of all ICA-extracted and GO-compiled signatures were tested using GSEA at E4, E6, E8, and E12 ($n = 4-6$), compared with nonpregnant (NP) mice ($n = 6$). The significantly enriched signatures ($FDR\ p < 0.05$) were used to generate functional modules using the Cytoscape software and its Enrichment Map plugin. Functional modules were manually circled and assigned a label based on GO annotation. Nodes represent molecular signatures, and their size is proportional to the number of genes composing the molecular signature; lines connecting nodes reflect mutual overlap between nodes. Shown in red are the upregulated signatures and in green are the downregulated ones. **(B)** Treg signature heat map showing the kinetics of a previously described Treg signature, that is, which is composed of genes that discriminate $\text{Foxp3}^+\text{CD25}^+$ cells from non-Tregs (49), at E4, E6, E8, and E12 ($n = 4-6$), compared with NP mice ($n = 6$). Shown in red are upregulated genes and in green are downregulated ones.

Antigen presentation pathway



fetuses and tolerance to tumor cells on a larger scale, we compared the global transcriptomic changes occurring in the uterus during pregnancy with those changes occurring in the tumor microenvironment during tumor growth. We first analyzed the changes in the

tumor microenvironment as we did for the uterus. Supervised analyses of DEGs revealed an early downregulation (as early as day 4) of immune-related pathways, including the Ag presentation pathway (Supplemental Fig. 3) and DC maturation (Supplemental

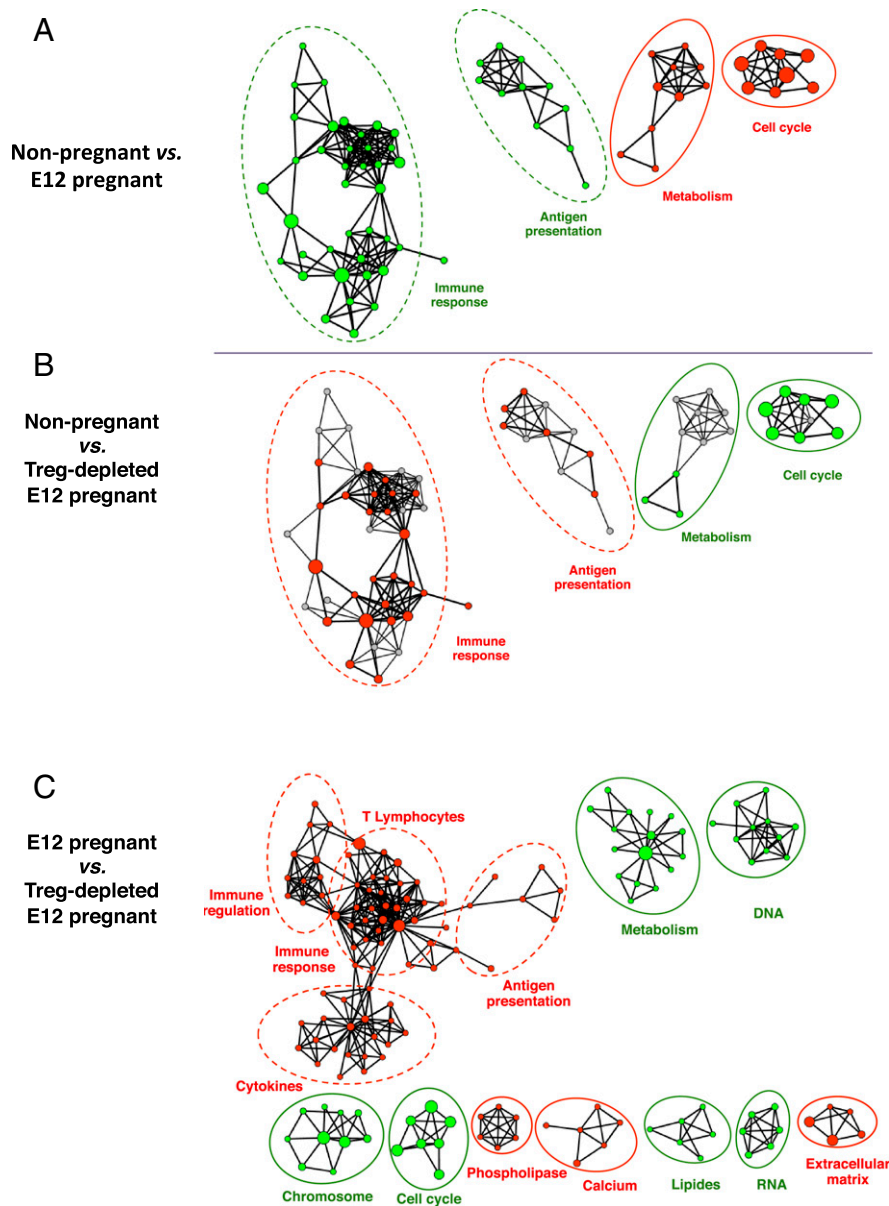


FIGURE 4. Effects of Treg depletion on the regulation of pathways in the pregnant uterine tissues. The signature modules were obtained as described in Fig. 2 and pathways regulated at E12 are shown. Shown in red are upregulated signatures and in green are the downregulated ones. Functional modules were manually circled and assigned a label based on GO annotation. Immune-associated modules are represented in dotted lines. Comparisons with nonpregnant mice of untreated (**A**) or Treg-depleted (**B**) mice are shown. (**B**) Shown in red are shared up-regulated signatures, in green shared down-regulated signatures, and in gray nonshared signatures. (**C**) Comparison between Treg-depleted and untreated pregnant mice. Data are representative of $n = 4$ –5 mice per group.

Fig. 4B). Unsupervised analyses showed that cell proliferation- and metabolism-related signatures were upregulated, whereas the immune response-annotated signatures were downregulated as early as day 4 after tumor cell inoculation, with increased changes over time (Fig. 5). Thus, the temporal and functional patterns of changes are quite similar in the cancer and pregnancy settings.

We then directly compared signature modulation between the pregnancy and cancer settings, revealing a strikingly similar pattern of gene regulation (Fig. 6). Most of the immune-related signatures that were rapidly downregulated in the pregnancy setting (Fig. 2A, nonpregnant versus E6) were also consistently downregulated in the cancer setting (Fig. 6A). This was also true at later time points, when more immune response-associated signatures were downregulated (Fig. 6B). At any time points between E4 and E12, there was not even a single downregulated immune response-related signature that was upregulated in the cancer setting.

We next analyzed all significantly enriched signatures (with the Benjamini-Hochberg FDR of <0.05) from the GO and KEGG

databases, whatever the biological function they represent, shared by the two conditions across all time points. We found a total of 37 common signatures (Fig. 6C). Strikingly, the only 10 upregulated signatures are all related to cell proliferation, whereas the 27 downmodulated ones are all associated with the immune response. Ten of these signatures are related to Ag presentation and six to T lymphocyte function. Among the other signatures, five are labeled as related to diseases, all being allo- or autoimmune diseases.

Overall, these studies reveal that within the complex microenvironment changes accompanying tumor and fetal development, the one and only striking overall similarity is the downmodulation of the immune response. This is true in terms of timing and of mechanisms, with decreases of Ag presentation and T cell activation, and increase of Treg activation. To strengthen the significance of these observations, we performed similarity analyses between up- and downregulated ICA extracted signatures from the two datasets. Jaccard and GOSemSim (36) analyses, which test the similarity of signatures in terms of gene composition and functional annotation, respectively, confirmed the high significance of

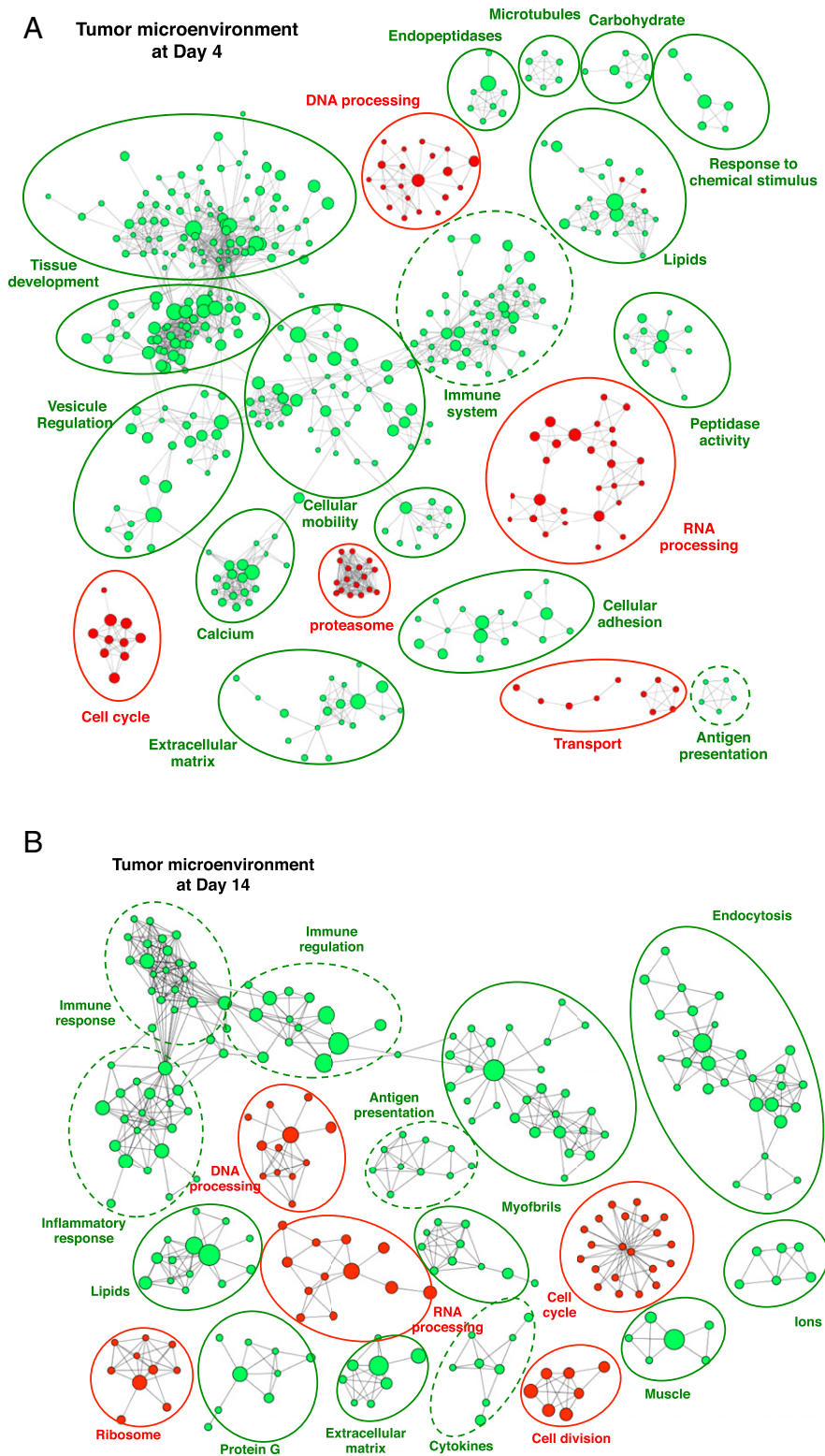


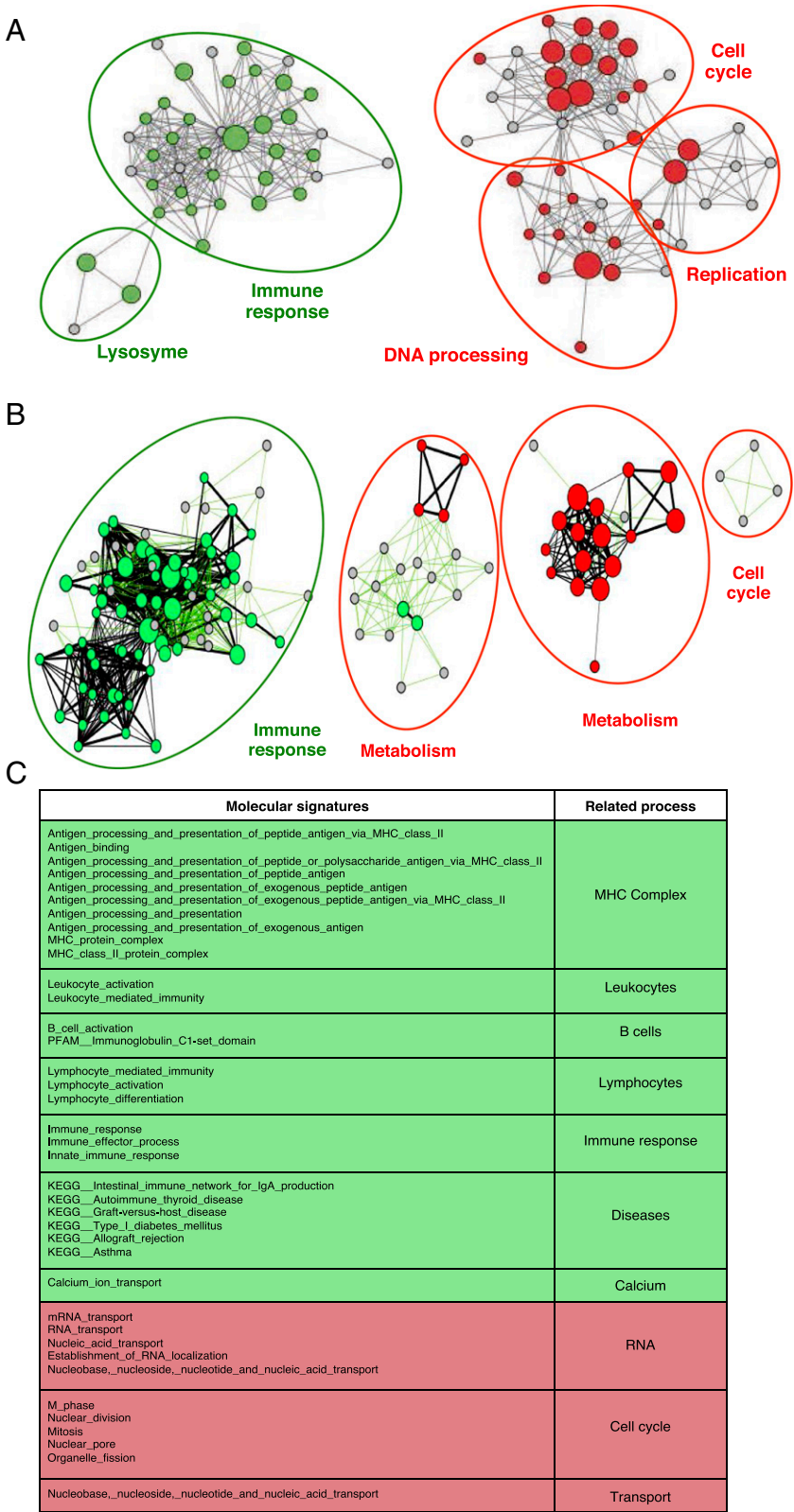
FIGURE 5. Unsupervised analyses of the dynamic regulation of functional pathways in the tumor microenvironment. The enrichments of all ICA-extracted and GO-compiled signatures were tested using GSEA. The significantly enriched signatures (FDR $p < 0.005$) were used to generate functional modules using the Cytoscape software and its Enrichment Map plugin as described in Fig. 2. Nodes represent molecular signatures, and their size is proportional to the number of genes composing the molecular signature; lines connecting nodes reflect mutual overlap between nodes. Shown in red are upregulated signatures and in green are the downregulated ones. (A) day 4 and (B) day 14 after tumor injection. Data are representative of $n = 4\text{--}7$ mice per group.

the above findings (Fig. 7A, Supplemental Fig. 2E). We then focused on the signatures that showed the highest Jaccard and GOSemSim indices, which correspond to those with the highest similarity between the cancer and pregnancy settings. Among the upregulated signatures, there were again those annotated as metabolism and cell cycle (data not shown). Among the downregulated signatures, there were again those associated with DC maturation, IFN signaling, the Ag presentation pathway, and IL-17 signaling (Fig. 7B).

Discussion

Since 1953, pregnancy has been viewed as nature's allograft (43), with the maternal immune system being in direct contact with a semiallogeneic organism, deeply engrafted and invasive, without any sign of rejection. Similarities between fetal and tumor development escaping the immune system were already alluded to >40 y ago (11). The authors concluded that an anti-inflammatory effect is a normal property of trophoblast cells and that "For a cell to become cancerous, it would have to combine a reappearance

FIGURE 6. Comparison of regulated pathways in the pregnant uterine tissues and tumor microenvironment. Comparison of significantly enriched signatures from uterine tissues and tumor microenvironment is shown. **(A)** E6 versus day 4; **(B)** E12 versus day 14. Shown in red are shared upregulated signatures, in green shared downregulated signatures, and in gray nonshared signatures. **(C)** Intersection of all signatures. All signatures significantly enriched (up- or down-regulated) from the tumor microenvironment (at day 4 and day 14) and uterine tissues (at E4, E6, E8, E10, E11, and E12) were compared. Shown in red is the list of shared upregulated signatures and in green are the downregulated ones.



of trophoblastic properties and at least another lesion altering the regulatory circuits controlling normal division.”

The following decades of studies concerning maternal–fetal or tumor cell immune tolerance have emphasized the numerous cell types and mechanisms involved in ensuring the success of pregnancy (9, 12, 44, 45) or the development of tumors (46, 47). However, as most of these studies were focused on individual molecules or cells, our understanding of maternal–fetal and cancer

immune tolerance remains fragmented. Understanding the global/operational nature of an immune response, such as in immune tolerance, requires the characterization of its individual components, but also the exploration of the complex interactions and regulatory associations between these components. In this regard, our transcriptome studies revealed that >2000 genes were already significantly modulated at E4, and >5000 from E8 onward. Systems biology has the potential to tackle this

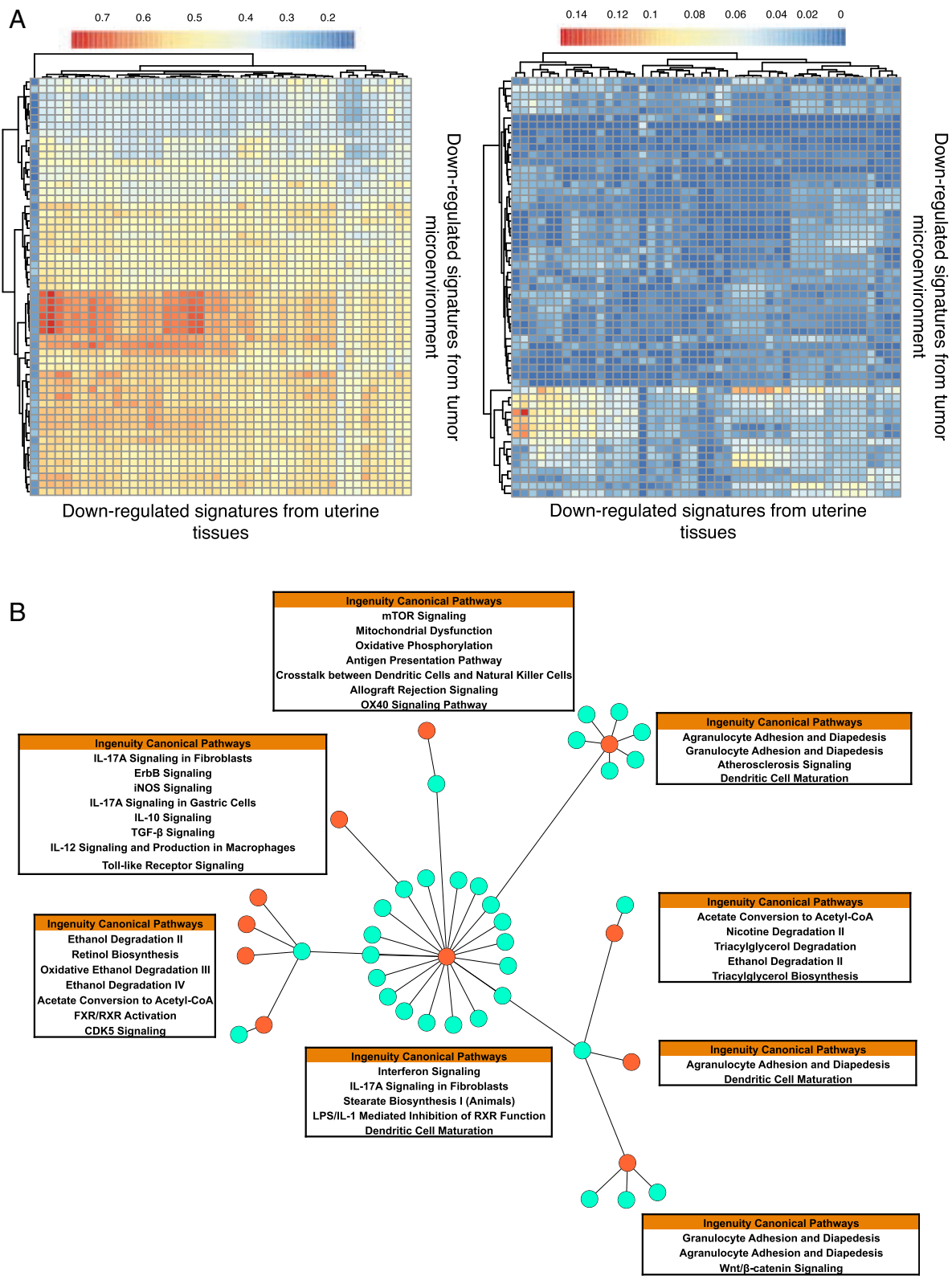


FIGURE 7. Similarity analysis of downregulated signatures from uterine tissues and the tumor microenvironment. **(A)** ICA-extracted downregulated signatures from uterine tissues (UT) and the tumor microenvironment (TM) were compared using the GOSemSim (*left panel*) and Jaccard (*right panel*) similarity indices. The heat map colors represent the similarity index as shown on the scale. The similarity indices range between 0 and 1. **(B)** Using Cytoscape software, signatures with the highest GOSemSim and Jaccard similarity indices were represented as a network. TM signatures are depicted in orange, UT signatures are shown in blue. TM signatures were then functionally annotated using IPA and the immune-related pathways were shown.

huge complexity. Furthermore, using non-hypothesis-driven studies, it has the potential to discover components beyond our current knowledge.

We analyzed the global fetal tissues and tumor microenvironment using whole transcriptome microarray and enrichment analyses with GO gene sets. The gene sets tested in our analyses

cover all the GO biological processes, which notably include developmental, cellular, metabolic, and immune system processes. The latter represent <3% of the entire GO database. It is thus remarkable that, besides genes related to the intense cell proliferation, the only genes revealed by our studies to be robustly modulated are immune response-associated genes, which were all downregulated in both settings (Figs. 2A, 5). Of note, in the cancer setting there were many more signatures that were modulated than in the physiological pregnancy setting (Fig. 5). In fact, many functions other than immune responses, such as cellular adhesion or cellular mobility, were downmodulated. This observation suggests that tumor cells may enlist further pathways to license their development.

At E8 (Fig. 2A) fewer immune-related signatures are represented as compared with both E6 or E12, whereas the total number of significantly modulated signatures is not affected. Because no variability issues were noted within the E8 group, we speculate that this could be due to a hormonal modulation of the immune system, as pregnancy-associated hormones can influence adaptive immune responses (48).

It is also remarkable that all the immune response signatures are downmodulated, whereas one would have expected upregulation of Treg-related signatures. Actually, this could not be the case, as no Treg signatures are represented in the GO database. We thus specifically analyzed the dynamic behavior of Treg signatures from the literature (40–42, 49) and observed that global immune-related signature downregulation coincides with the upregulation of previously published Treg signatures (Fig. 2B, Supplemental Fig. 2A–D). Altogether, these results reveal that immune downmodulation is a hallmark of the pregnancy and cancer microenvironments.

The detailed analysis of the immune-related pathways involved in tumor and fetal protection revealed important similarities. Among others, our studies highlight that Ag presentation, lymphocyte activation, and Treg activation are the central downmodulated pathways (Fig. 3, Supplemental Figs. 3, 4B). Furthermore, IPA annotation of highly similar ICA-extracted signatures from tumor microenvironment and uterine tissues revealed the presence of genes belonging to the DC maturation pathway in almost all shared signatures (Fig. 7B). These findings support the involvement of immature DCs in the establishment of tolerogenic microenvironments, and they are in line with the importance of the cross-talk between Tregs and DCs for maintenance of tolerance (12). Treg-mediated suppression of DC function is mediated at least in part by the interaction of CTLA4 on Tregs with CD80/CD86 on DCs. This results in downregulation of CD80/CD86 expression and defective Teff activation (50, 51), which we saw in our transcriptomic dataset and confirmed by flow cytometry analyses of uterine DCs (Supplemental Fig. 4A). These results indicate that uterine tissues are enriched in immature DCs upon embryo implantation. Such an increase in immature DCs has been described in human decidua in early pregnancy (52). In mice, the number of immature DCs also increases in normal pregnancy following implantation, whereas an increase in mature DCs was reported in pregnant female mice mated in abortion-prone conditions (53). Furthermore, a recent study reported a detailed and quantitative analysis of uterine leukocyte populations from E5.5 to E16.5 of pregnancy, the results of which are in line with our observations (54).

Although systems studies can highlight the importance of specific molecules such as CD80/CD86, our results also emphasize that it would be pointless to try to reduce maternal–fetal immune tolerance to one or a few major molecules/pathways. Actually, we think that further understanding of maternal–fetal immune toler-

ance will require a systems biology approach on an even larger scale, incorporating, for example, metabolic or microbiota studies, both locally and in the whole organism.

Supervised DEG analysis of uterine tissues identified two waves of immune responses over time: an early one, likely triggered by embryo implantation, and a later one during midgestation (Fig. 1D, Supplemental Fig. 1C). For the early wave, most gene expression changes started at day 6 after fertilization (E6). As embryo implantation takes place at around E4.5, this suggests that physical contact between the embryo derivatives and the uterine tissue is important in triggering immune response modulation. Nevertheless, enrichment analysis using molecular signatures pointed to downregulation of immune-related signatures as early as E4, that is, before embryo implantation (Fig. 2A). Thus, unsupervised purely statistical generation of signatures appears more sensitive than supervised methods and may reveal early changes of immune-annotated signatures that could be related to Treg activation by seminal fluid (55). A recent study demonstrated that a soluble form of CD38 released from seminal vesicles induces tolerogenic DCs and activates Tregs, thereby enhancing maternal immune tolerance and protecting the fetus from rejection (56).

Similar early downmodulation of immune response genes is also observed in the cancer setting. These results confirm the brisk Treg recruitment and activation triggered by embryo or tumor cell implantation (16, 21). This brisk response is due to the mobilization of self-specific memory Tregs, the response of which is faster than that of naive Teffs, and thus predates and pre-empt the Teff response. We also observed a significant modulation of myeloid-derived suppressor cell signatures (57) in the tumor microenvironment, suggesting the involvement of this subset in immune tolerance to tumors (data not shown).

The second wave of downmodulated immune pathways is not so strikingly different, and includes activation marker-related pathways (CD40, 4-1BB, CTLA4) and cytokine pathways (IL-1, IL-9, IL-10, IL-17). It could correspond in part to the recruitment of induced peripheral Tregs, which have also been reported to play an important role in successful pregnancy (58).

The depletion of many cell types mobilized to support normal pregnancy does not result in abortion (10). In contrast, this is the case for Tregs, both thymic Tregs (21, 22) and induced peripheral Tregs (58). This highlights the very peculiar and nonredundant role of Tregs in successful pregnancy. In our experiments, the depletion of Tregs reversed the downmodulation of immune response genes not just to the baseline state of a nonpregnant uterus, but to an activated state where the same immune signatures that were downmodulated are now upregulated compared with the nonpregnant uterus. This emphasizes the control imposed by Tregs on effector immune responses that would develop otherwise. The combined analyses of the cellular and molecular immune response are compatible with a scenario in which the early recruitment of self-specific memory Tregs imprints a tolerogenic environment by both tuning the DCs toward a tolerogenic functional state and directly suppressing Teffs.

The first viviparous mammals were faced with the development of a sophisticated adaptive immune system (12). The development of placentation in eutherians required the co-development of immune suppression mechanisms. We and others have hypothesized that the selection of Tregs during evolution, whether natural thymic Tregs (21) or peripheral-induced Tregs (58), has been mostly driven by the need to protect the fetus. Our present study, using unsupervised transcriptome studies, confirms the similarities in downregulation of effector immune response during pregnancy and cancer, orchestrated by Tregs.

The American writer Susan Sontag had the prescience to describe her tumor as a “demonic pregnancy.” “This lump is alive,” “a fetus with its own will” she wrote in *Illness as Metaphor* (59). Thirty-eight years later, her literary vision has been validated by systems biology. We now think that the protection of cancer cells by Tregs became the price paid for the evolution of an efficient protection of embryos. Pregnancy and cancer represent two aspects of immune tolerance, physiological and pathological, respectively. Comparison of these settings is heuristic and identifies shared tolerogenic signatures. Further study of these signatures should now help discover targets for immune intervention to improve or to break immune tolerance. As Tregs are central to both cancer and maternal–fetal tolerance, their manipulation has therapeutic potential. Treg depletion in cancer should help break the tolerogenic environment and improve the efficacy of immunotherapies. In contrast, stimulating Tregs may help control recurrent spontaneous abortion of immune origin. We recently showed that low-dose IL-2 is a safe and specific Treg inducer in humans (60, 61) and that it could prevent recurrent spontaneous abortion in the DBA2/CBA2 model in mice (25). Thus, low-dose IL-2 warrants investigation in recurrent spontaneous abortions and infertility caused by implantation failure.

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Disclosures

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