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Immunopathology and Infectious Diseases

Increased Expression of Peripheral Blood Leukocyte Genes Implicate CD14$^+$ Tissue Macrophages in Cellular Intestine Allograft Rejection

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Recurrent rejection shortens graft survival after intestinal transplantation (ITx) in children, most of whom also experience early acute cellular rejection (rejectors). To elucidate mechanisms common to early and recurrent rejection, we used a test cohort of 20 recipients to test the hypothesis that candidate peripheral blood leukocyte genes that trigger rejection episodes would be evident late after ITx during quiescent periods in genome-wide gene expression analysis and would achieve quantitative real-time PCR replication pre-ITx (another quiescent period) and in the early post-ITx period during first rejection episodes. Eight genes were significantly up-regulated among rejectors in the late post-ITx and pre-ITx periods, compared with nonrejectors: *TBX21*, *CCL5*, *GNLY*, *SLAMF7*, *TGFBR3*, *NKG7*, *SYNE1*, and *GK5*. Only *CCL5* was also up-regulated in the early post-ITx period. Among resting peripheral blood leukocyte subsets in randomly sampled nonrejectors, CD14$^+$ monocytes expressed the CCL5 protein maximally. Compared with nonrejectors, rejectors demonstrated higher counts of both circulating CCL5$^+$CD14$^+$ monocytes and intragraft CD14$^+$ monocyte-derived macrophages in immunohistochemistry of postperfusion and early post-ITx biopsies from the test and an independent replication cohort. Donor-specific alloreactivity measured with CD154$^+$ T-cytotoxic memory cells correlated with the *CCL5* gene and intragraft CD14$^+$ monocyte-derived macrophages at graft reperfusion and early post-ITx. *CCL5* gene up-regulation and CD14$^+$ macrophages likely prime cellular ITx rejection. Infiltration of reperfused intestine allografts with CD14$^+$ macrophages may predict rejection events. ***(Am J Pathol 2011, 179:1929–1938; DOI: 10.1016/j.ajpath.2011.06.040)***

Recurrent rejection limits long-term patient and graft survival after intestinal transplantation (ITx) in children. Recurrent rejection occurs in the majority of the 30% to 60% ITx recipients who experience early acute cellular rejection (ACR), and less commonly in those who are rejection-free early after ITx. Within 5 years after ITx, 15% of recipients have progressed to chronic rejection and graft loss.[1,2] The association between early and recurrent rejection suggests that the early rejector who develops recurrent episodes and the recipient who is rejection-free early after transplantation represent two contrasting outcome groups in which to study the mechanisms of early and progressive allograft injury. The high incidence of recurrent rejection after ITx has not been eliminated with high-dose prophylactic immunosuppression with anti-T-cell agents or aggressive surveillance with frequent protocol biopsies. High-dose T-cell immunosuppression leads to life-threatening infections and post-transplant lymphoma-like disorders in nearly one fifth of all recipients.[2,3] Novel mechanisms must be sought to better di-

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rect preventive immunosuppression and to identify the rejection-prone intestine allograft recipient.

Recent studies suggest that the enhanced T-cell-mediated and non-T-cell-mediated immune mechanisms that characterize rejection can also be seen during periods of quiescence in rejection-prone recipients, and may trigger recurrent episodes of rejection. First, higher numbers of circulating natural killer (NK) cells are seen in addition to T-cytotoxic cells (Tc) before ITx and during rejection episodes.[4] Second, enhanced donor-specific alloreactivity measured by allospecific CD154$^+$ T-cytotoxic cells (TcM) can be detected before ITx and for several weeks before and after biopsy-proven rejection.[5,6] CD154$^+$ TcM are highly sensitive and specific for biopsy-proven rejection. Third, a relative excess of myeloid dendritic cells, which may have inflammatory functions, can be seen before and after rejection of cadaveric intestine allografts.[7] Also, rejection after living related ITx is preceded by enhanced interferon (IFN) α signaling, which likely emanates from and also implicates circulating dendritic cells.[8] Fourth, rejection-prone ITx recipients exhibit decreased expression of defensins by intestinal epithelial cells during periods of quiescence, and higher frequencies of risk alleles in the *NOD2* gene, which have previously been associated with defective innate immunity in Crohn's disease.[9–11] None of these studies evaluated potential mechanisms of recurrent rejection that might be evident during periods of quiescence between rejection episodes and that could precipitate acute ITx rejection, nor have such potential mechanisms been linked to early rejection, as is suggested by the high likelihood of recurrent rejection among children who experience early rejection.

We describe a peripheral blood molecular signature from rejection-prone ITx recipients that consists of several up-regulated inflammatory genes serving innate and adaptive immune pathways. Among these, an up-regulated *CCL5* gene appears to be a strong candidate for priming ITx rejection via CD14$^+$ monocyte-derived macrophages (MDM). Allografts destined to experience rejection exhibit increased infiltration with CD14$^+$ MDM within hours after reperfusion.

Materials and Methods

All study procedures were performed after approval by the University of Pittsburgh's Institutional Review Board.

Study Design

Forty-one children with ITx were studied (Figure 1): 20 in a test cohort, 18 children in two validation cohorts (9 each), and 3 additional children with chronic rejection and graft loss. Candidate gene discovery with microarrays during a quiescent rejection-free period late after ITx, quantitative real-time PCR replication of candidate genes at additional time periods, and early identification of candidate cells associated with rejection were conducted in blood and biopsy tissue from a test cohort of 20 children. An independent validation cohort of nine children with ITx provided allograft biopsies for validation of the candidate cell type with immunohistochemistry. Rejectors were defined as children who experienced two or more episodes of biopsy-proven ACR, the first within 90 days after ITx. Nonrejectors did not experience ACR during this time period.

Discovery of candidate peripheral blood leukocyte (PBL) genes was performed with Affymetrix (Santa Clara, CA) 1.0 ST whole-genome exon arrays. These blood samples were obtained late after ITx, at least 90 days removed from an ACR event, during a period of quiescence. Expression characteristics of candidate genes were confirmed with quantitative real-time PCR in resid-

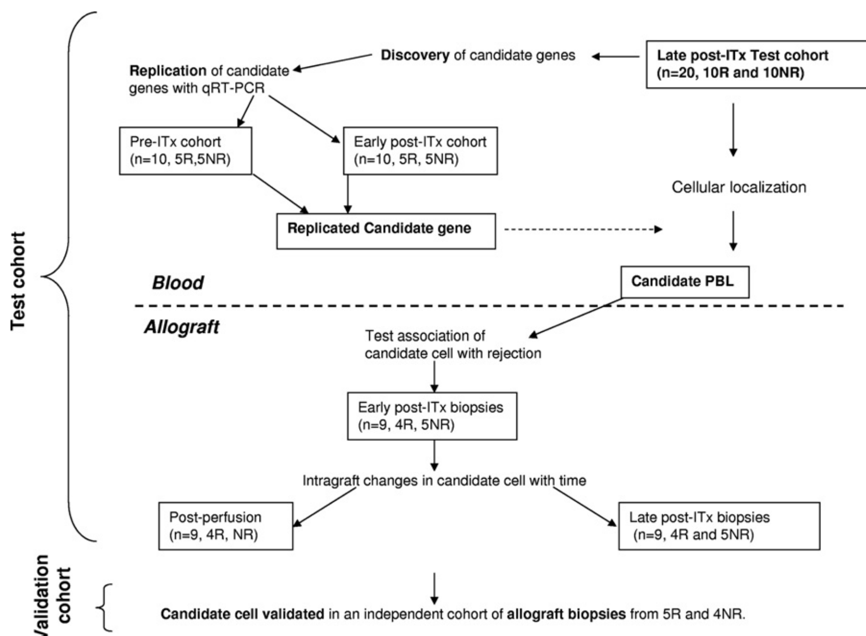

Figure 1. Study design. Twenty-nine children were evaluated in the present study, 20 in the test cohort and 9 in an independent validation cohort. The test cohort of 20 children provided i) late post-ITx blood samples for candidate gene discovery with microarrays, ii) pre-ITx and early post-ITx blood samples for quantitative real-time PCR replication of candidate genes, iii) random blood sample for cellular localization of the replicated candidate gene, and iv) allograft biopsy tissue to identify the candidate cell associated with rejection along with its time-dependent changes in postperfusion and late post-ITx allograft biopsies. Postperfusion biopsies are obtained within 4 hours of allograft implantation, and within 24 hours of pre-ITx blood sampling. All other biopsies correspond to blood samples used for gene expression studies. An independent validation cohort of nine children provided additional biopsy tissue to confirm the time course of the candidate cell associated with ITx. NR, nonrejector; R, rejector.

ual RNA from the same subjects. Quantitative real-time PCR replication of candidate genes was performed in the pre-ITx and early 90-day post-ITx time period when first rejection episodes occur. Samples from these two time periods were obtained from a subset of children in the test cohort ($n = 10$). Candidate genes replicated at all time periods were localized (using random, late post-transplant blood samples from a subset of children in the test cohort; $n = 9$) to PBL subsets that exhibited maximal expression of the corresponding protein. The role of the candidate PBL in ACR was first evaluated with single-color IHC in allograft biopsies corresponding to the early post-ITx period, when ACR is first seen. Time-dependent changes in the candidate cell type were further evaluated in postperfusion biopsies obtained within 24 hours of pre-ITx blood samples, and in biopsies corresponding to the late post-ITx periods in the test cohort. Finally, the candidate cell and its time-dependent changes were validated with IHC in postperfusion biopsies, and in biopsies in the early post-ITx and late post-ITx periods, in an independent validation cohort of children with ITx ($n = 9$).

The relevance of the candidate PBL gene and the intragraft candidate cell in ITx rejection was evaluated further by testing correlations with allospecific $CD154^+$ T-cytotoxic memory cells ($CD154^+$ TcM). Allospecific $CD154^+$ TcM were measured in mixed-lymphocyte reaction (MLR) of corresponding blood samples at all time periods. Expressed as the immunoreactivity index IR or the ratio of donor- to third-party-induced $CD154^+$ TcM, the cell type has a sensitivity of 93% and specificity 88% for association with ITx rejection if the IR is $\geq$1.23.[5]

Peripheral blood corresponding to biopsies in the validation cohort was consumed in other studies. Therefore, peripheral blood candidate genes were also replicated in blood samples obtained during a quiescent rejection-free period late after ITx in an additional test cohort of nine recipients (four rejectors, five nonrejectors). The relationship between the replicated candidate gene and chronic rejection was further evaluated during a quiescent period later after transplantation in three additional test children who experienced graft loss due to chronic rejection.

Human Subjects

At the time of ITx, children either received the polyclonal rabbit, anti-human thymocyte globulin (rATG; Genzyme, Cambridge, MA), or the monoclonal alemtuzumab (Genzyme, Cambridge, MA), as described previously.[1,2,11] Both agents contain antibody specificities directed against a variety of PBL subsets, including T and B cells. Alemtuzumab was given to the more recent ITx recipients, to reduce the incidence of early ACR in children, based on recent success in adult ITx recipients.[1] All children received tapering doses of steroids, and the T-cell immunosuppressant tacrolimus titrated to whole blood concentrations (FKWBC) 10 to 15 ng/mL during the first 90 days, and tapered to 5 to 10 ng/mL toward the end of the first year.[2,10] The frequency of protocol biopsies was weekly in the first month, twice monthly during the second month, and monthly thereafter until closure of the allograft stoma. The grade and severity of ACR was determined by one of two pathologists (S.R. and R.J.). Criteria were increased crypt apoptosis and destruction, lamina propria infiltration with lymphocytes, with or without ulceration, as described previously.[12] ACR was defined as either steroid-responsive or steroid-resistant (if additional lymphocyte depletion therapy was needed).

Study Procedures

RNA was extracted from 2.5-mL blood samples collected in PAXgene tubes (PreAnalytiX-Qiagen-BD, Hombrechtikon, Switzerland), using the manufacturer's protocol for automated extraction on a QIAcube system (Qiagen, Valencia, CA). Isolated total RNA (1 μg) was reverse-transcribed to cDNA for TaqMan gene expression assays (Applied Biosystems, Foster City, CA) for the following genes: granulysin (GNLY, HS01120727_M1), transforming growth factor, beta receptor III (TGFBR3, Hs01114253_M1), chemokine (C-C motif) ligand 5 (CCL5, HS00174575_M1), SLAM family member 7 (SlamF7, HS00904275_M1), glycerol kinase 5 (GK5, HS01074700_M1), natural killer cell group 7 (NKG7, HS00366585_G1), spectrin repeat containing, nuclear (SYNE1, HS00941968_M1), T-box 21 (TBX21, HS00894394_G1). Target mRNA levels are expressed relative to an internal glyceraldehyde-3-phosphate dehydrogenase control (GAPDH, Hs99999905_m1; Applied Biosystems). For cDNA amplification, PCR was performed on a model 7300 real-time PCR system (Applied Biosystems), with 10 minutes incubation at 95°C to activate AmpliTaq Gold DNA polymerase; this was followed by 40 cycles with 15 seconds at 95°C and 1 minute at 60°C for each cycle. Data were analyzed using Sequence Detector System (SDS) software version 1.3.1. (Applied Biosystems).

Whole-genome expression identified candidate genes in the blood as those achieving preset fold change of $\geq$1.2 or $\leq$1.2 and $P < 0.02$ in a case-controlled comparison of 10 rejectors and 10 nonrejectors, as described previously.[13] Quantitative real-time PCR replication was performed with TaqMan probes (Applied Biosystems). For analysis, the cycle threshold (Ct) value and the relative Ct method was used. All Ct values were normalized to the expression of the housekeeping gene *GAPDH* for three technical replicates for each sample. Rejectors and nonrejectors were compared in each cohort to determine significance at $P < 0.05$, and to determine whether the changes matched those predicted in discovery. Final results were expressed as the fold change for each gene in rejectors, compared with nonrejectors, using the formula Relative Expression $= 2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct_{Rejector\ group} - \Delta Ct_{Nonrejector\ group}$. Pathway analysis of differentially expressed genes was performed with a Web-based algorithm from Ingenuity Systems, Redwood City, CA.

Flow Cytometry

Flow cytometry and intracellular staining with monoclonal fluorochrome labeled antibodies was used to measure resting protein expression of *CCL5* in $CD14^+$ monocytes (the circulating precursors of intestinal macrophages), $CD4^+$ T-helper cells, $CD8^+$ T-cytotoxic cells, $CD19^+$ B

cells, and CD56$^+$/CD16$^+$ NK cells. Donor-specific alloreactivity was measured in flow cytometric mixed-lymphocyte reaction assays, as described previously.[5] Flow cytometry antibodies were CCL5-phycoerythrin, CD14-allophycocyanin-Cy7, CD4-phycoerythrin-Cy5, CD8-phycoerythrin-Cy7, CD56/CD16-fluorescein isothiocyanate, CD154-phycoerythrin (BD Biosciences, San Jose, CA), and CD19-Texas Red (Beckman Coulter, Miami, FL). CD45RO-Texas Red was obtained from Beckman-Coulter.

Tissue Immunohistochemistry

Sequential slides were re-cut into tissue sections (4 μm thick) from stored formalin-fixed, paraffin-embedded tissue blocks for nine children in the test cohort. After antigen retrieval with CC1 at 100°C for 32 minutes (Ventana Medical Systems, Tucson, AZ), slides were stained in batches on a Ventana immunostainer (DAKO M7050 at 1:100, Ventana CCI mild, 32 minutes at 32°C, iView 3,3′-diaminobenzidine detection) as described previously.[13] Primary antibodies were to CD163 (mouse monoclonal; Vector Laboratories, Burlingame, CA) a generic macrophage marker, CD14 (rabbit monoclonal; Vector Laboratories), and CCL5 (mouse monoclonal; Lifespan Biosciences, Seattle, WA). Peroxidase-labeled secondary antibodies were used as final stain. Positive and negative controls were run for each staining batch and stain. Hematoxylin counterstain was applied after 3,3′-diaminobenzidine detection. Cell counting was performed blinded to outcome, and before a second histological review of the tissues. Duplicate counts were made on each 10th case to assess variance. The lamina propria was evaluated for the total number of stained cells and the cell count per high-power field (40× objective) was determined as an average of five fields for each stain. Tissue IHC was validated on sequential biopsies from nine additional children in the validation cohort, using primary antibodies to CD14.

Statistical Analysis

The Affymetrix human 1.0 ST exon microarray was used to measure transcript-level differential gene expression in peripheral blood, as described previously.[11] Briefly, probe summaries were computed using the Affymetrix Power Tools (APT) software version 1.10.1 and the rma-sketch normalization method. Principal components analysis and correlation heatmaps were used to identify outliers. (None were identified; data not shown.) For the PBL gene comparison in rejectors and nonrejectors, both a fold change and a Welch's modified *t*-test were calculated for each transcript cluster probe set. Significance was set at $P < 0.05$.

Results

For the 41 children evaluated in the present study, the median age was 1.8 years (range, 0.9 to 21), the male:female distribution was 20:21, and the rejector:nonrejector distribution was 23:18. The distribution of treatment rATG:alemtuzumab was 37:4, and of transplant type isolated ITx:combined liver-ITx was 18:23. Blood samples were obtained at a median (±SEM) interval of 0 days, 26 ± 14 days (range, 17 to 56), and 453 ± 143 days (range, 127 to 2357) for the pre-ITx, early post-ITx, and late post-ITx cohorts in the test and validation cohorts. Rejectors experienced a median of three ACR episodes (range, 3 to 7), with the first episode occurring at 14 ± 4.7 days (mean ± SD) and the most recent episode occurring at 623 ± 766 days after ITx. Among the 23 rejectors in the present study, one child experienced multiple episodes of mild ACR; all others experienced at least one episode of moderate ($n = 13$) or severe ($n = 9$) rejection. Five of 23 rejectors went on to experience chronic rejection and graft loss. Nineteen of 23 rejectors required antilymphocyte therapy at least once for steroid-resistant ACR Among nonrejectors, no ACR was observed during the first 90 days. In two nonrejectors, a single episode of mild ACR occurred at 627 and at 822 days after ITx.

A Molecular Signature of Recurrent Rejection Based on PBL Genes

Among genes with significant differential expression ($P <$ 0.05; see Supplemental Table S1 at *http://ajp.amjpathol.org*), eight genes achieved threshold ≥1.2-fold expression and $P <$0.02 in rejectors compared with nonrejectors in PBL using genome-wide gene expression (Table 1) and technical replication with quantitative real-time PCR in the late post-ITx test cohort. These eight genes were also replicated in an additional cohort of nine test recipients who were also evaluated during quiescence late after ITx. The genes were granulysin (*GNLY*), transforming growth factor, beta receptor III (*TGFBR3*), chemokine (C-C motif) ligand 5 (*CCL5*), SLAM family member 7 (*SLAMF7*), glycerol kinase 5 (*GK5*), natural killer cell group 7 (*NKG7*), spectrin repeat containing, nuclear (*SYNE1*), and T-box 21 (*TBX21*). Up-regulation in rejectors was replicated for all eight genes in the pre-ITx cohort, and only for *CCL5* in the early post-ITx cohort (Figure 2A). The respective Ct value differences between groups are presented in Supplemental Figure S1 and Supplemental Table S2 (available at *http://ajp.amjpathol.org*). To evaluate whether CCL5 up-regulation preceded or was associated with chronic rejection, RNA from three additional children who experienced graft loss due to chronic rejection was evaluated with quantitative real-time PCR. Mean ± SD ΔCt values significantly higher among chronic rejectors, compared with nonrejectors (1.14 ± 0.38 versus 2.26 ± 1, $P = 0.021$). A lower ΔCt implies higher gene expression and vice versa.

Ingenuity pathway analysis (Ingenuity Systems, Redwood City, CA) using the above-mentioned genes as a query identified CCR5-CCL5 cytokine-receptor signaling in macrophages as the top-ranked canonical pathway on the basis of the *CCL5* gene alone, whereas antigen presentation was identified as the top-ranked biological function on the basis of the *CCL5*, *TBX21*, *GNLY*, and *TGFBR3* genes (Figure 3A).

Table 1. Significantly Up-Regulated Candidate Genes Among Rejection-Prone Children

Gene symbol	Gene name	FC	*P* value	Function
GNLY	Granulysin	2.14	0.0085	Cytotoxic Th, Tc, and NK cells
NKG7	Natural killer cell group 7 sequence	2.06	0.0074	NK cell receptor, macrophages/monocytes activated Th, Tc
CCL5	Chemokine (C-C motif) ligand 5	1.98	0.013	Macrophage activation, chemotaxis, Tc, Th
SLAMF7	SLAM family member 7	1.79	0.0051	NK cell function, B-cell activation
TGFBR3	Transforming growth factor, beta receptor III	1.51	0.015	Growth, development, Wnt/β-catenin signaling
SYNE1	Spectrin repeat containing, nuclear envelope 1	1.45	0.0075	Expressed on smooth muscle and PBL, no previous association
GK5	Glycerol kinase 5 (putative)	1.36	0.0016	Not known
TBX21	T-box 21	1.25	0.0032	Th, Tc, NK cells, promotes renewal of memory cells

Changes in expression showing FC > 1.2 and $P < 0.02$ during rejection-free intervals (Affymetrix 1.0 ST gene arrays). Down-regulated genes did not achieve these thresholds of expression.
FC, fold change; NK, natural killer; Tc, T-cytotoxic; Th, T-helper.

The CD14$^+$ Monocyte-Derived Macrophage As a Candidate Cell in ITx Rejection

Five PBL subsets were tested with intracellular cytokine staining in random blood samples obtained during the late post-ITx period from five rejectors and four nonrejectors. The resting expression of *CCL5* was highest in CD14$^+$ monocytes from nonrejectors, compared with Tc and NK subsets (Figure 2, B and C). Also, CCL5$^+$CD14$^+$ monocytes were significantly higher in rejectors compared with nonrejectors ($P = 0.006$; Figure 2, B and C). The expression of *CCL5* in Th and B cells did not approach that seen in monocytes, Tc, and NK cells (data not shown).

Allograft biopsies corresponding to blood samples from the early post-transplant period from four rejectors and five nonrejectors in the test cohort were evaluated with single-color IHC. CD14$^+$ MDM per high-power field were significantly higher in biopsies from children with ACR, compared with those which were rejection-free (23 ± 8 versus 3 ± 0.9, $P = 0.017$) (Figure 3B). Numerically higher counts of CD163$^+$ macrophages per high-power field (32 ± 3 versus 27 ± 11, $P > 0.05$) and CCL5-stained cells per high-power field (26 ± 6 versus

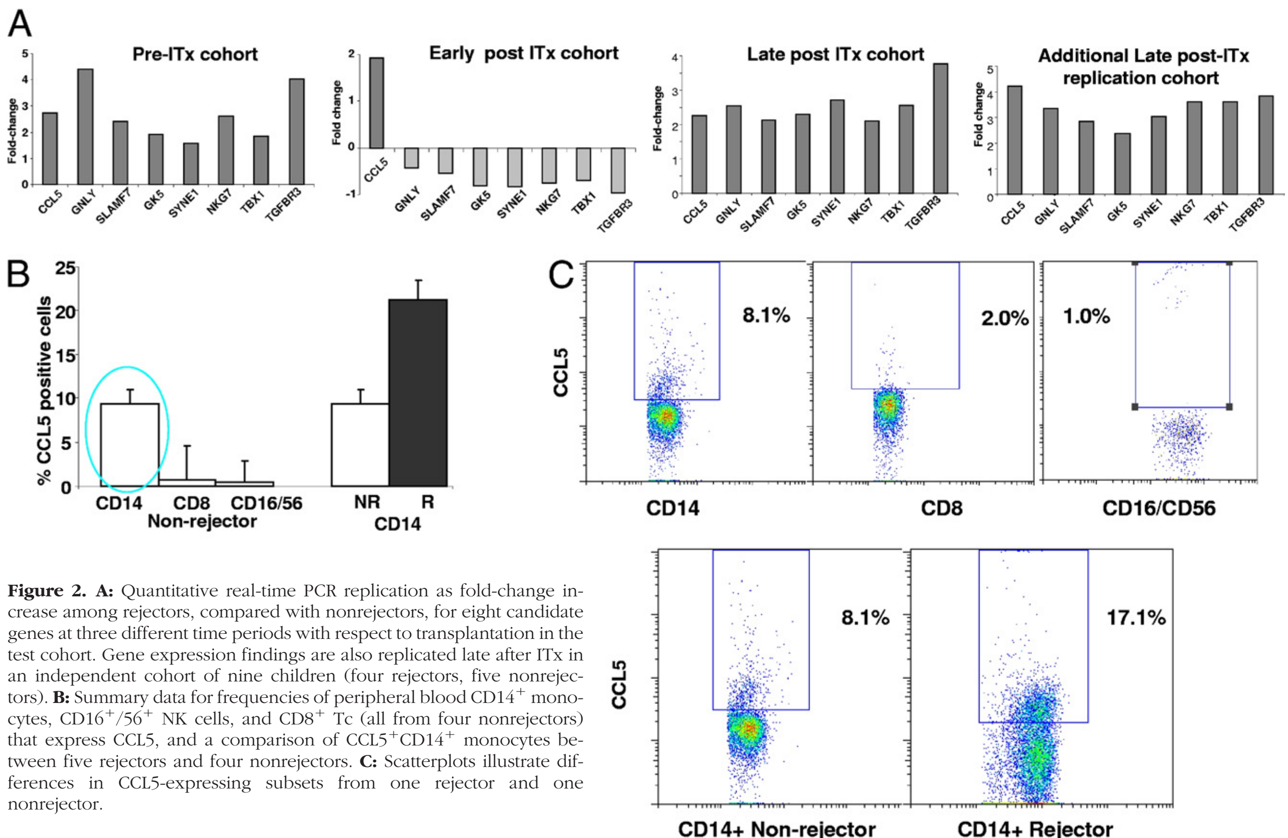

Figure 2. A: Quantitative real-time PCR replication as fold-change increase among rejectors, compared with nonrejectors, for eight candidate genes at three different time periods with respect to transplantation in the test cohort. Gene expression findings are also replicated late after ITx in an independent cohort of nine children (four rejectors, five nonrejectors). **B:** Summary data for frequencies of peripheral blood CD14$^+$ monocytes, CD16$^+$/56$^+$ NK cells, and CD8$^+$ Tc (all from four nonrejectors) that express CCL5, and a comparison of CCL5$^+$CD14$^+$ monocytes between five rejectors and four nonrejectors. **C:** Scatterplots illustrate differences in CCL5-expressing subsets from one rejector and one nonrejector.

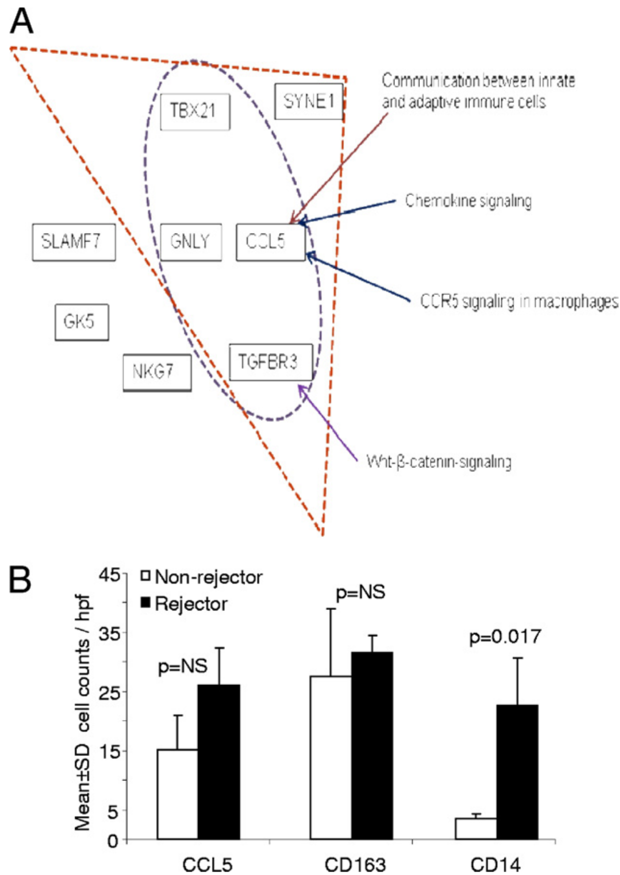

Figure 3. **A:** Canonical pathways (**arrows**), antigen-presentation as a top-ranked biological function (enclosed within **oval**), and inflammatory disease as one of the top-ranked disorders (enclosed within **triangle**) suggested by different combinations of candidate genes in Ingenuity Systems pathway analysis. **B:** Significantly higher intragraft $CD14^+$ MDM counts per high-power field in allograft biopsies from rejectors, compared with rejection-free biopsies from nonrejectors. No statistical differences were seen for $CD163^+$ macrophages and CCL5-stained cells per high-power field.

15 ± 6, $P > 0.05$) were also observed in biopsies showing ACR, but the difference did not achieve statistical significance in between-group comparisons (Figure 3B). Of note, CCL5 staining was present in the nucleus and the cytoplasm and also generated high background staining, making $CCL5^+$ cell counts less reliable. Therefore, the remaining allograft biopsies from the test ($n = 9$) and validation ($n = 9$) cohorts were stained only for $CD14^+$ MDM.

In the test cohort, $CD14^+$ MDM counts were significantly higher in postperfusion allograft biopsies (12 ± 2 versus 2 ± 2, $P = 0.005$) and in early post-ITx biopsies with ACR (23 ± 8 versus 3 ± 0.9, $P = 0.017$) from rejectors, compared with nonrejectors (Figure 4A). No such differences were observed in the late post-transplant period (6 ± 6 versus 3 ± 3, $P > 0.05$). These time-dependent changes in intragraft $CD14^+$ MDM were mirrored by similar changes in immunoreactivity index of allospecific $CD154^+$ TcM in blood samples corresponding to allograft biopsies in the test cohort (Figure 4B). Increased intragraft $CD14^+$ MDM counts per high-power field among rejectors were also seen in postperfusion (23 ± 17 versus 4 ± 3, $P = 0.038$) and early post-ITx biopsies showing ACR (20 ± 11 versus 5 ± 5, $P = 0.045$) in the validation cohort (Figure 4, C and D), compared with nonrejectors. No such between-group differences were seen in the late post-ITx period (7 ± 6 versus 2 ± 2, $P > 0.05$).

Inflammatory Genes and Intragraft Macrophages Correlate with Donor-Specific T-Cell Alloreactivity

A lower ΔCt value for a gene indicates up-regulation. A significant negative correlation was seen between the ΔCt for CCL5 and donor-specific alloreactivity measured by the immunoreactivity index of $CD154^+$ TcM in the pre-ITx (Spearman's $r = -0.88$, $P = 0.004$) and in the early post-ITx cohorts (Spearman $r = -0.65$, $P = 0.04$). Significant positive correlations were also seen between allospecific $CD154^+$ TcM in the pre-ITx blood sample and intragraft macrophage counts in postperfusion allograft biopsies (Spearman's $r = 0.81$, $P = 0.05$). These significant correlations between $CD154^+$ TcM and $CD14^+$ MDM were also seen in the early post-ITx cohort ($r = 0.82$, $P = 0.003$) (see Supplemental Figure S2 at *http://ajp.amjpathol.org*). No such correlations were seen in the late post-ITx period. Summary data further demonstrated that the immunoreactivity of allospecific $CD154^+$ TcM was significantly increased in rejectors compared with nonrejectors during the pretransplant and early post-transplant periods (Table 2).

Discussion

Our search for mechanisms that initiate recurrent cellular rejection of intestine allograft identifies several up-regulated genes. These genes, which mediate innate and adaptive inflammatory functions of several types of PBL, were identified during a period of quiescence late after ITx. Functions include activation and chemotaxis for macrophages, lymphocytes, and dendritic cells (*CCL5*, *TBX21*, *GNLY*); effector and memory differentiation of T cells and terminal differentiation of NK cells (*TBX21*); and cytotoxicity (*GNLY*, *SLAMF7*).[14–22] *NKG7* is expressed on T cells, NK cells, and macrophages, and up-regulation has previously suggested an important role for the macrophage in renal allograft rejection.[23,24] Circulating TGFβR3 binds to TGFβ and facilitates its binding to TGFβR1 and TGFβR2 to mediate downstream signaling.[25] The functional significance of *SYNE1* and *GK5* has not been fully established in the immune response. These eight genes were selected from microarray data on the basis of arbitrarily chosen thresholds of expression, because our small sample of the rare pediatric ITx recipient population did not allow multiple testing corrections. However, all genes achieved technical replication with quantitative real-time PCR in residual RNA from samples that were used for discovery. These genes were also up-regulated before ITx, suggesting that early and recurrent rejection may be primed by similar mechanisms. However, only *CCL5* met our pre-defined criteria for up-regulation at all time periods after ITx, because it was

Figure 4. Sequential changes in intragraft CD14$^+$ MDM per high-power field (**A**) and the immunoreactivity index of allospecific CD154$^+$ TcM (**B**) in peripheral blood in rejectors and nonrejectors from the test cohort. **C:** Changes in intragraft CD14$^+$ MDM per high-power field in serial allograft biopsies from the validation cohort. **D:** Composite of serial hematoxylin-eosin stained biopsies with peroxidase stained CD14$^+$ MDM (dark brown) from one nonrejector and one rejector. Magnification is 40×. ACR, acute cellular rejection; hpf, high-power field.

also up-regulated in blood samples drawn in proximity to allograft biopsies showing rejection.

Failure to replicate all genes in early post-ITx samples may have occurred because, during rejection, the cellular sources of the remaining seven genes may have been sequestered at the site of (allograft) rejection in recipients in whom lymphocyte-depleting immunosuppression may have depleted peripheral blood cell reserves. Alternatively, high-dose immunosuppression used to treat rejection may have inhibited the expression of several genes. However, only one of five rejectors was sampled after initiation of treatment for rejection. The ΔCt of 1.56 for *CCL5* expression in this rejector was higher than the highest expression seen among nonrejectors (ΔCt range, 1.68 to 3.17), but was within the range seen in the remaining rejectors (1.02 to 1.99). A lower ΔCt implies higher gene expression and vice versa. Increased *CCL5* expression shortly after high-dose antirejection treatment further supports the importance of this gene and its associated pathway in priming rejection events, and is also consistent with its up-regulation in rejection-prone children during periods of relative immunological quiescence, despite varying induction regimens. Two rejectors and two nonrejectors in the test cohort were induced with alemtuzumab. The remainder were induced with rATG. The average ΔCt for CCL5 in alemtuzumab or rATG-treated rejectors (0.39 versus 0.95, respectively) and nonrejectors (2.5 versus 2.2, respectively) was similar.

Additional studies were aimed at defining a cellular source of CCL5 that might mediate allograft injury. In

Table 2. Immunoreactivity Indices of Allospecific CD154$^+$ T-Cytotoxic Memory Cells Are Higher in Rejectors Compared with Nonrejectors, in Children

Immunoreactivity index of CD154TcM in children with CCL5 gene expression					Immunoreactivity index of CD154TcM in children with IHC studies for CD14+MDM				
	n	Pre ITx	*n*	Early post ITx		*n*	Pre ITx	*n*	Early post ITx
NR	5	0.388 ± 0.06	4	0.502 ± 0.13	NR	4	0.486 ± 0.16	6	0.512 ± 0.12
R	4	2.03 ± 0.67	6	3.99 ± 3.9	R	2	1.22 ± 0.09	4	4.71 ± 5.8
P		0.0158		0.009	*P*		0.042		0.009

Mann-Whitney test was used.
ITx, intestinal transplantation; MDM, monocyte-derived macrophages; NR, nonrejectors; R, rejectors.

these studies, the $CD14^+$ monocyte-derived macrophage emerges as the best candidate cell to initiate ITx rejection for several reasons. First, CCL5 is maximally expressed in the relatively unstimulated or understimulated $CD14^+$ monocytes from nonrejectors, compared with Tc and NK cells, and is significantly higher in $CD14^+$ monocytes from rejectors (Figure 3). Also, the *CCL5* gene is up-regulated in blood samples from rejectors for all three time periods after ITx. This up-regulation corresponds with elevated intragraft content of $CD14^+$ MDM in the pretransplant and early post-transplant periods in rejectors (Figure 2). Second, pathway analysis identifies CCR5 signaling in macrophages as the top-ranked canonical pathway based on the *CCL5* gene. Along with *GNLY*, *TBX21*, and *TGFBR3*, *CCL5* also identifies antigen presentation as the top-ranked biological function (Figure 3). Macrophages are known to present antigen. Third, we found significantly higher $CD14^+$ MDM counts in allograft biopsies showing rejection, compared with those that are rejection-free (Figure 4). Of note, counts for macrophages that express the generic macrophage activation marker CD163 and CCL5-stained cells were numerically higher in biopsies from rejectors, but did not achieve statistical significance. As in other inflammatory intestinal diseases, changes in the normal $CD14^-$ gut macrophage population during acute inflammation are likely dependent on infiltration of the gut with inflammatory blood-monocyte-derived $CD14^+$ macrophages.[26–28] During health, $CD14^+$ monocytes replenish normal gut macrophages, but acquire the anergic profile of resident gut macrophages.[29,30] Finally, sequential biopsies in the test and validation cohorts had significantly higher $CD14^+$ MDM counts within 4 hours after graft reperfusion and during ACR among rejectors. These elevated counts normalize later after ITx to levels seen among nonrejectors. The $CD14^+$ macrophage thus plays an important role in initiating ITx rejection.

Our findings suggesting a role for the $CD14^+$ macrophage in ITx rejection have several parallels. In experimental models, ITx rejection is not eliminated by the absence of any single T-cell subset, suggesting that other types of inflammatory cells may be responsible.[31–33] More to the point, macrophage depletion of the ITx recipient or the donor, alone or in combination, is known to attenuate experimental ITx rejection.[34] Among clinical ITx recipients, calprotectin (a macrophage product released during intestinal inflammation) has shown promise in the diagnosis of ITx rejection.[35] In renal transplant rejection, macrophages have been identified in the inflammatory infiltrates of rejection in several studies since 1975.[36,37] Finally, the acute intestinal inflammation in inflammatory bowel diseases is characterized by replacement of the $CD14^-$ resident gut macrophage with the inflammatory $CD14^+$ MDM in several previous reports.[26–28]

The role of the CCL5-macrophage axis in priming ITx rejection is also supported by the significant correlations demonstrated by this PBL gene and intragraft $CD14^+$ MDM with allospecific $CD154^+$ TcM at the time of ITx and during rejection. Allospecific $CD154^+$ TcM measure donor-specific alloreactivity, which is uniformly enhanced with allograft rejection.[5,38] Our previous studies also demonstrate that the rejection threshold of $CD154^+$ TcM, if present before ITx, has a sensitivity of 100% and specificity of 90% for prediction of early ITx rejection.[6] In the present study, significant correlations are seen between this cell type and peripheral blood *CCL5* gene expression before ($P = 0.004$) and after ITx ($P = 0.042$). Reasonably good correlations are also seen between $CD154^+$ TcM and intragraft $CD14^+$ MDM at the time of ITx in postperfusion allograft biopsies ($P = 0.051$), and during ITx rejection ($P = 0.003$), despite the small numbers of subjects available for evaluation. These observations support our contention that the CCL5-macrophage axis has an important role in initiating ITx rejection.

Despite up-regulation of the peripheral blood *CCL5* gene during all time periods before and after ITx, the intragraft localization of its corresponding protein has remained challenging and can be seen as a limitation of the present study. This occurred in part because of the technical difficulties posed by the high background staining of formalin-fixed, paraffin-embedded biopsy tissue (Figure 3B). However, previous studies also demonstrate that CCL5 is expressed in undifferentiated blood monocytes, promotes migration of these monocytes to tissues, but is repressed in the migrated monocyte as it transforms into the tissue macrophage.[39] These data may also explain why the CCL5 protein was not identified consistently in the allograft. Future studies in snap-frozen tissue may resolve this limitation of the present study.

That allograft infiltration by blood-derived monocyte/macrophage is an early event in intestine allograft rejection is not the only conclusion from the present study. The larger utility of our work is the possibility that a coordinated innate and adaptive immune response likely produces both early and late intestine allograft injury. Among rejection-prone recipients, molecular signals arising from both effector arms (represented by the up-regulated genes in the present study) are present before ITx, along with enhanced donor-specific alloreactivity. This signal is also present late after resolution of ITx rejection, when enhanced donor-specific alloreactivity has abated among rejectors (Figure 4B). Late allograft injury primed by these signals may be one reason for progression to chronic rejection and eventual graft loss, which characterizes 15% of all ITx recipients.[2] To illustrate, 2 of 10 rejectors tested late after transplantation with microarrays lost their allografts because of chronic rejection. *CCL5* expression was greatly increased in these children. Also, three additional children with chronic rejection exhibited CCL5 overexpression during a quiescent late post-transplant period. Together, this evidence supports the idea that acute, recurrent, and chronic allograft injury may have common mechanisms. Known therapy that can target monocytes (which exhibited high resting expression of CCL5 in rejectors) is limited and includes steroids, which were administered at the time of graft perfusion in all patients. Despite this approach, reperfused allografts destined to experience rejection exhibited early infiltration with $CD14^+$ MDM, suggesting that higher doses or protracted pretransplant administration may be beneficial. Prospective validation studies by our research group are ongoing.

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