Nuclear CD40 interacts with c-Rel and enhances proliferation in aggressive B-cell lymphoma

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CD40 is an integral plasma membrane–associated member of the TNF receptor family that has recently been shown to also reside in the nucleus of both normal B cells and large B-cell lymphoma (LBCL) cells. However, the physiological function of CD40 in the B-cell nucleus has not been examined. In this study, we demonstrate that nuclear CD40 interacts with the NF-κB protein c-Rel, but not p65, in LBCL cells. Nuclear CD40 forms complexes with c-Rel on the promoters of NF-κB target genes, CD154, Blys/BAFF, and Bfl-1/A1, in various LBCL cell lines. Wild-type CD40, but not NLS-mutated CD40, further enhances c-Rel–mediated Blys promoter activation as well as proliferation in LBCL cells. Studies in normal B cells and LBCL patient cells further support a nuclear transcriptional function for CD40 and c-Rel. Cooperation between nuclear CD40 and c-Rel appears to be important in regulating cell growth and survival genes involved in lymphoma cell proliferation and survival mechanisms. Modulating the nuclear function of CD40 and c-Rel could reveal new mechanisms in LBCL pathophysiology and provide potential new targets for lymphoma therapy. (Blood. 2007;110:2121-2127)

Introduction

CD40 is a cell membrane receptor protein of the tumor necrosis factor receptor (TNF-R) superfamily that functions in B lymphocytes for B-cell activation, antigen presentation, immunoglobulin isotype switching, and development of germinal centers and humoral immune memory.1 Besides B lymphocytes, CD40 is expressed in dendritic cells,2 monocytes and macrophages,3 and a variety of nonhematopoietic cells.4-6 The binding of CD40 to its cognate ligand, CD154 (CD40L), is required for initiating thymus-dependent humoral responses in the immune system.7 Ligation and subsequent triggering of the CD40 plasma membrane receptor lead to a variety of immune and inflammatory responses.8 When CD40L binds to its receptor, CD40 optimally signals as a dimeric or trimeric aggregate from its location in plasma membrane microdomains known as lipid rafts.9-11 In the lipid rafts, CD40 recruits multiple TNF receptor–associated factors (TRAFs), an important group of intracellular adaptor molecules that bind directly or indirectly to cytoplasmic domains of various members of the TNF receptor superfamily,12,13 and the IKK complex, forming a macromolecular signaling complex called the CD40 signalingosome.14 Through CD40 ligand cognate receptor binding, this signalosome transmits signals via the NF-κB pathway to promote cell proliferation and survival and mediate immune and inflammatory responses.14,15

Constitutively activated NF-κB pathway is a molecular signature characteristic of many aggressive B-cell lymphoma cells.16 The CD40 signalingosome pathway has also been shown to be important in modulating normal and neoplastic B-cell proliferation and survival, primarily through the activation of NF-κB target genes, such as CD40L17, Blys18, and Bfl-1/A1.19,20 CD40L is endogenously expressed in aggressive B-cell lymphomas, binding to the CD40 receptor, and constitutively activates the canonical NF-κB signaling pathway.17 Blys signaling activates noncanonical NF-κB signaling pathway21,22 and promotes cell survival, cell-cycle progression and proliferation of B cells,23-26 and large B-cell lymphoma (LBCL).18 Bfl-1/A1, an antiapoptotic member of the Bcl-2 family, whose expression is confined primarily to the immune system, also plays an important role in cell survival.27,28 Agents that selectively block CD40-NF-κB pathway have been shown to inhibit cell growth and induce apoptosis in aggressive B-cell lymphoma cells.29

Among the NF-κB family members activated by CD40 pathway, only c-Rel has been shown to be able to transform cells in the lymphoid cell lineage and has been identified as a bona fide oncogene.30 Amplifications of human c-rel are frequently seen in Hodgkin lymphoma, some follicular and mediastinal B-cell lymphomas, and LBCL.31 The molecular mechanism of c-Rel involves its transcriptional function in NF-κB–regulated gene promoters.17,18 Our studies have demonstrated that c-Rel is a pivotal transcription factor that regulates CD40 responsive genes, such as CD40L and Blys,17,18 through interaction with another important lymphoid transcription factor, NFATc1. Recent studies have also shown that c-Rel exerts its function through recruiting multiple transcription factors, forming a macromolecular enhanceosome in the promoter of Bfl-1/A1.32 However, the precise composition and function of a putative c-Rel enhanceosome in LBCL remain to be characterized.

Recent findings from our lab indicate that the CD40 receptor is also present in the nucleus of both normal and neoplastic human B cells, adding CD40 into a list of membrane receptors whose nuclear localization has revealed a novel mode of action for these receptors, including epidermal growth factor receptor (EGFR) and fibroblast growth factor receptor (FGFR).33-36 We also identified...
the BLyS/BAFF promoter as a molecular target for nuclear CD40, suggesting an important transcriptional role of CD40 as well as its relationship to the BLyS/BAFF system, however, the molecular mechanism and physiological function of nuclear CD40 are still elusive. We hypothesized that nuclear CD40 may interact with certain transcription factors on the promoters of B-lineage cell growth– and survival-associated genes.

In this study, we show that nuclear CD40 interacts with c-Rel but not with p65, another NF-κB member. This specific interaction forms transcriptional complexes on the promoters of c-Rel target genes, activating gene expression, and enhancing normal and neoplastic B-cell proliferation.

Materials and methods

Cells

Human LBCCL cell lines (MS, McA, and FN) were established from diagnostic biopsy tissue or effusions from patients as previously described.18 This study was conducted in accordance with the Helsinki protocol and approved by the M. D. Anderson Cancer Center institutional review board. Informed consent was obtained from all patients in accordance with the declaration of Helsinki. The cells were cultured in RPMI (Gibco, Rockville, MD) containing 10% fetal calf serum (HyClone, Logan, UT). Normal human B lymphocytes were purified from healthy donors by using the human B-cell enrichment cocktail from StemCell Technologies (Vancouver, BC) as described previously.14 The resulting cell populations were stained 95% to 98% positive for CD20 and analyzed by flow cytometry. Fresh biopsy-derived lymphoma tissues (patients 1-5) were minced in cold RPMI and single-cell suspensions of lymphoma cells were purified by Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ), and stained positive for CD19, CD20, CD10, but negative for CD3, by immunohistochemistry.

Plasmids and reagents

The 6xNF-κB CD40LTR/Km reporter was obtained by cloning 6 copies of the NF-κB binding site (5’-AGGAGTTTCCA-3’) of the CD40L promoter upstream of the minimal thymidine kinase promoter in the pGL3 luciferase reporter plasmid19 (a gift from Dr Peter Vandenbergh, Laboratory for Experimental Hematology, University of Leuven, Belgium). The pCMV-c-Rel expression vector was a gift from Dr Celine Gelin20 (Center for Advanced Biotechnology and Medicine, Piscataway, NJ). The pCMVp65 expression vector was a gift from Dr Nancy Rice21 (National Cancer Institute-Frederick, Frederick, MD). The pcDNA3.1/myc/his-CD40 and pcDNA3.1/myc/his-CD40NLS mutant plasmids were constructed by cloning the polymerase chain reaction (PCR) product generated using the pGL4-basic promoter as a molecular target for nuclear CD40, the NF-κB/H11032 B binding site is underlined) was labeled with [32P]ATP. Nuclear extract (10 μg) was incubated 1× binding buffer, 2 μg polyclonal, and 1 μL labeled probe (4 fmol/μL) at 37°C for 15 minutes. The samples were then subjected to 5% nondenaturing acrylamide gel electrophoresis and visualized with x-ray film. For supershift analysis, 1 μL antibody was added to the mixture 15 minutes before the labeled probe was added. Unlabeled oligonucleotide (400 fmol) was used to compete with the labeled probe.

Electrophoretic mobility shift assays

Nuclear protein extraction and gel shift assay procedures were performed as previously described.14,40 Briefly, a double-stranded NF-κB consensus oligonucleotide 5’-AGT TGA GGC GAC CCT TTT CCC AGG-3’ (Promega, Madison, WI; NF-κB binding site is underlined) was labeled with [γ-32P]ATP. Nuclear extract (10 μg) was incubated 1× binding buffer, 2 μg polyclonal antibody, 0.5% nonidet P-40, and 1 μL labeled probe (4 fmol/μL) at 37°C for 15 minutes. The samples were then subjected to 5% nondenaturing acrylamide gel electrophoresis and visualized with x-ray film. For supershift analysis, 1 μL antibody was added to the mixture 15 minutes before the labeled probe was added. Unlabeled oligonucleotide (400 fmol) was used to compete with the labeled probe.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChiP) assays were performed using the ChiP assay kit and protocol provided by the manufacturer (Upstate Biotechnology) and as described previously.17 DNA fragments were purified for polymerase chain reaction (PCR) amplification with PCR beads from Amersham Pharmacia Biotech (Piscataway, NJ). Oligonucleotide primers specific for the CD154 promoter (forward, 5’-CACCCTCCTGGGAGTGATTATCA-3’; reverse, 5’-GATAATACACGATGGTCCTC-3’), the BLyS promoter (forward, 5’-GAGACAGAATCTTCTC-GGACTGATAAAG-3’; reverse, 5’-GACCCGGTTGCACTT TTCTCTTCT-3’; reverse, 5’-GGTAAATCCCCGTCTGTACTAA-3’). The PCR conditions were as follows: the cDNA template was denatured at 95°C for 1 minute, annealed at 50°C for 30 seconds, and extended at 72°C for 1 minute per cycle for 35 cycles. The PCR product was visualized on a 2.0% agarose gel.
Transfection, luciferase, and β-galactosidase assays

Transient transfections of cultured lymphoma cells and normal B lymphocytes were conducted using the nucleofector protocol from Amaza Biosystems (Cologne, Germany). Luciferase and β-galactosidase (β-gal) assays were performed according to the manufacturer’s directions (Promega). Each sample in an experiment was repeated at least 3 times. Luciferase activity values were normalized to transfection efficiency monitored by the cotransfected β-gal expression vector.

In vitro [³H]thymidine incorporation assay

In vitro thymidine incorporation proliferation assays were performed as described previously.²¹ Cells were plated as triplicate wells in 200 μL RPMI 10% FCS, with indicated antibody reagents in a 96-well culture plate and incubated in 5% CO₂/air at 37°C. After 24 hours, each well was pulsed with 0.5 μCi (0.0185 MBq)/10 μL [³H]thymidine (Amersham Pharmacia Biotech) and incubated for 16 hours; then, cells were harvested and counted in a Beckman LS3800 liquid scintillation counter (Hialeah, FL).

Results

CD40 interacts with c-Rel in the nucleus of LBCL cells

Recently, we have reported that CD40 can enter the nucleus in normal and neoplastic B cells. Nuclear CD40 binds to the promoter of BLyS gene and up-regulates BLyS expression. Because BLyS is also a target gene of c-Rel, we speculated that CD40 might interact with c-Rel in the nucleus. We tested this hypothesis using a coimmunoprecipitation assay. We chose 2 large B-cell lymphoma (LBCL) cell lines (MS, McA) previously established in our lab. Nuclear fractions from these cells were incubated with CD40 antibody and coimmunoprecipitation analyses were performed (Figure 1A). When CD40 was precipitated, c-Rel was also coimmunoprecipitated in both cell lines. These data indicated a physical association between CD40 and c-Rel. However, we did not detect p65 in these experiments, suggesting the interaction between CD40 and c-Rel is specific. The presence of Oct-1 and the lack of β-actin in the nuclear fraction indicate that the nuclear fraction is free of cytosolic contamination. To further confirm the interaction between CD40 and c-Rel, we used coimmunoprecipitation with c-Rel and c-reg (Figure 1B). Confocal microscopy also showed that CD40 (green) and c-Rel (red) both colocalized (yellow) in the nucleus of LBCL cells (Figure 1C), indicating that CD40 interacts with c-Rel in the nucleus of large B-cell lymphoma cells.

Nuclear CD40 enhances c-Rel–mediated BLyS promoter activation in LBCL cells

Since c-Rel is a well-characterized transcription factor, we addressed the question of whether CD40 is involved in the transactivation of c-Rel through their interaction. Here, we used a luciferase pGL3 reporter plasmid (6xNF-κB-CD40L/TkM) containing 6 copies of the CD154-NF-κB binding site oligonucleotide inserted upstream of the thymidine kinase minimal promoter. To emphasize the nuclear function of CD40, we used both wild-type CD40 (CD40wt) and nuclear localization signal (NLS) mutated CD40 (CD40NLSmut) constructs. Because CD40 cannot enter the nucleus after its NLS is mutated, CD40NLSmut loses its nuclear function. The NLS motif of CD40 is a distinct signature that does not overlap with the TRAF binding sites in the CD40 protein²⁸; therefore, the CD40NLSmut should still share the same membrane function with CD40wt. After transfection of the reporter plasmid along with CD40wt, CD40NLSmut, or c-Rel, luciferase activity was measured (Figure 2A). C-Rel activated the promoter through its cognate binding (14.5-fold increase), while CD40wt and CD40NLSmut both up-regulated this reporter through their membrane function-activation of NF-κB (14.3-fold and 11.4-fold increases, respectively). The combination of c-Rel and CD40wt synergized reporter activation (47.9-fold increase); however, this synergy was diminished with the cotransfection of c-Rel and CD40NLSmut (20.2-fold increase). Because CD40NLSmut does not enter the nucleus, this synergy represents a nuclear function of CD40.

Since BLyS is a common target for both c-Rel and CD40, we tested whether overexpression of exogenous c-Rel and CD40 proteins would lead to either additive or synergistic BLyS promoter activity in LBCL cells, and whether CD40 would play differential roles between c-Rel and p65 on BLyS promoter activity. Cotransfection of the BLyS-luc reporter plasmid containing BLyS promoter region along with expression vectors containing c-Rel, p65, CD40wt, or CD40NLSmut resulted in differential activation of BLyS promoter activity (Figure 3B). Overexpression of c-Rel or CD40wt increased BLyS promoter activity by 6.4- and 4.1-fold, respectively, and combined c-Rel and CD40wt overexpression...
resulted in a synergistic 15.6-fold activation of the \textit{BlyS} promoter. However, combined c-Rel and CD40NLSmut overexpression diminished this synergy (5.1-fold increase). In the case of p65, overexpression of p65 alone increased \textit{BlyS} promoter activity by 3.2-fold, while both CD40wt and CD40NLSmut enhanced p65 40% up-regulated activity similarly on \textit{BlyS} promoter (7.2-fold and 9.3-fold increases, respectively). Because p65 did not interact with CD40 in the nucleus and CD40NLSmut does not enter the nucleus, these experiments demonstrated that CD40 interacts with c-Rel in the nucleus of LBCL cells and nuclear CD40 potentiates c-Rel transactivity through the specific interaction of these proteins.

\textbf{CD40 forms complexes with c-Rel on the promoters of proliferation- and survival-associated genes in lymphoma cells}

To investigate whether CD40 and c-Rel form a complex that binds to DNA, we synthesized the consensus NF-κB binding oligonucleotide and performed gel shift assays. When nuclear extracts from LBCL (MS) cells were incubated with this probe, a protein-DNA complex was visualized on a denaturing gel (Figure 3A) and the specificity of the binding was confirmed by competition with the excess (100×) unlabeled oligo probe. A supershift was detected with addition of c-Rel antibody (lane 4) and a decreased binding was seen with addition of CD40 antibody (lane 5). c-Rel and CD40 antibodies together further shifted the complex (lane 6). This result suggests that CD40 and c-Rel bind together with the NF-κB consensus binding site.

To further examine whether CD40 is involved in the transcriptional regulation of c-Rel in vivo, we performed chromatin immunoprecipitation (ChIP) assay to test whether CD40 and c-Rel form complexes on the promoters of c-Rel target genes. \textit{BlyS}, \textit{CD154}, and \textit{Bfl-1/A1} are important c-Rel target genes. The promoters of these genes were precipitated by c-Rel antibody and amplified by PCR as expected as positive controls, while normal IgG was used as negative control (Figure 3B). Interestingly, the same promoters were also precipitated by CD40 antibody. Both LBCL cell lines (MS and McA) confirmed the result and a negative control promoter from β-actin was shown at bottom. These data suggest that CD40 and c-Rel can form complexes on the promoters of c-Rel target genes including \textit{CD154}, \textit{BlyS}, and \textit{Bfl-1/A1}.

\textbf{Nuclear CD40 enhances c-Rel proliferation capacity in LBCL}

Next, we asked whether proliferation in lymphoma B cells is functionally enhanced by nuclear interaction between CD40 and c-Rel. To answer this question, thymidine incorporation assays were performed to detect the proliferative characteristics in LBCL cells (FN cells) after different transfections (Figure 4). As shown in Figure 4A, the combination of c-Rel and CD40wt showed almost a 200% increase in proliferation, when compared with c-Rel transfection alone, while the combination of c-Rel and CD40NLSmut resulted in a significantly smaller (90%) increase. Because CD40NLSmut does not enter the nucleus, these data indicate that the nuclear interaction of CD40wt and c-Rel augments proliferation in LBCL cells. To clarify the mechanism of this proliferation difference between CD40wt and CD40NLSmut, Western blotting was performed (Figure 4B). Overexpression of exogenous c-Rel and CD40wt and CD40NLSmut were detected by c-Rel antibody and Myc-tag antibody, respectively. Corresponding to the proliferation assay, cotransfection of c-Rel with CD40wt but not CD40NLSmut markedly up-regulated the expression of \textit{BlyS}, \textit{CD154}, and \textit{Bfl-1/A1}, indicating the nuclear CD40 enhanced the proliferative role of c-Rel through up-regulating its target genes.

\textbf{Nuclear expression of CD40 and c-Rel correlates with the proliferation of normal B lymphocytes}

Because neoplastic B cells share many similarities with the activated normal B cells, we addressed the status of nuclear CD40 and c-Rel in the normal B cells. We isolated normal B lymphocytes from healthy donors and extracted cytosolic and nuclear fractions, and Western blots were performed (Figure 5A). Upon B-cell activation, nuclear CD40 was significantly expressed in addition to cytoplasmic CD40, and c-Rel was fully activated, compared with quiescent G0 B cells. Normal B cells were transfected with either the empty vector or vector containing c-Rel and then activated with or without CD40 ligand, and then assayed for cell proliferation...
Stimulation of vector-transfected cells with CD40 ligand enhanced the proliferation of normal B cells (9.6-fold increase). Overexpression of c-Rel further enhanced the ability of CD40 to increase the proliferation of normal B cells (22.4-fold increase). These studies indicate that proliferation of normal activated B cells correlates with nuclear expression of CD40 and c-Rel.

While CD40 and c-Rel are expressed and interact in the nucleus of LBCL cell lines, the presence of these proteins is also shown in the patient lymphoma cell specimens in Figure 6. LBCL patient tumor cells were fractionated and Western blot analysis was performed. As shown in Figure 6A, CD40 and c-Rel are both consistently expressed in the nucleus of these patient lymphoma cells. Confocal microscopy also showed CD40 (green) and c-Rel (red) colocalized (yellow) in the nucleus of patient lymphoma cells (Figure 6B), suggesting the nuclear CD40 and c-Rel also function in LBCL patient cells.

Discussion

In this study, we found that the TNF receptor, CD40, interacts with the NF-κB transcriptional factor c-Rel in the nucleus of large B-cell lymphoma (LBCL) cells. Consistent with our previous findings, CD40 and c-Rel form a transcriptional complex in the promoters of NF-κB target genes including BLyS. Interaction of c-Rel with nuclear CD40 promotes proliferation in LBCL cells.

Nuclear translocation of cell membrane receptors is not limited to CD40. Recent studies have identified a growing list of growth factor receptors, including EGFR and FGFR family members, whose nuclear localization has been described in a variety of normal and neoplastic eukaryotic cell types.34-36,42 Functional studies on these nuclear localized plasma membrane–associated growth factors have focused largely on gene transcription processes. ErbB2/HER-2 has been shown to interact with the COX-2 promoter,35 and EGFR interacts with STAT3 and activates the iNOS/NO pathway.43 Our recent demonstration that CD40 is present in the nucleus of normal and neoplastic B cells raised questions regarding its function in the cell nucleus.33 In this study, our demonstration of the nuclear functional interaction between CD40 and c-Rel is of particular interest because CD40, a TNF receptor, not only activates both canonical and noncanonical NF-κB pathways through
classical membrane signaling, but also enhances the transactivation of NF-κB through nuclear localization and interaction with specific transcription factors in neoplastic B cells. As shown in Figures 2 and 4B, when the NLS of CD40 was mutated, it lost the ability to enter the nucleus, and mutated CD40 could not further up-regulate the expression of c-Rel target genes. The difference between plasma membrane and nuclear function of CD40 has also been shown in the proliferation experiments with LBCL cells (Figure 4A), in which fully functional CD40wt has both cell membrane and nuclear capability to mediate cell proliferation, but CD40NLSmut has lost this nuclear role. Many characteristics of activated normal B cells are retained in lymphoma B cells. Upon mitogenic activation, CD40 protein entered the nucleus in normal peripheral B lymphocytes (Figure 5 and Lin-Lee et al33) where c-Rel is also actively expressed in the nucleus. Concurrent nuclear CD40-c-Rel expression in normal B cells mimics the constitutive activation of CD40-NF-κB pathway in many LBCLs. Under such conditions, cell proliferation was stimulated and further enhanced by overexpression of c-Rel and CD40 ligand stimulation. This correlation of proliferation and nuclear accumulation of CD40 and c-Rel suggests that these 2 proteins also form a similar nuclear complex that functions in the nucleus of normal B lymphocytes.

c-Rel is particularly important among the NF-κB family members because of its oncogenic transformation capacity in chicken splenocytes and its amplification in some lymphoma patients. Our study, which has revealed the nuclear interrelationship between CD40 and c-Rel, suggests involvement of nuclear CD40 in lymphomagenesis. Nuclear localization of c-Rel has been demonstrated in several clinical settings. Correlation of c-Rel with other important molecular markers, such as amplification of REL locus as well as expression of TRAF1 and STAT1, has been shown as a diagnostic tool to identify classical Hodgkin lymphoma and mediastinal large B-cell lymphoma. Our finding that both CD40 and c-Rel are coexpressed constitutively in the nucleus of LBCL patient cells (Figure 6) suggests a clinical relevance for our findings.

Rel family proteins have a particularly strong propensity to function as components in macromolecular enhanceosomes by recruiting other transcription factors and cofactors into specific promoters, such as best-studied interferon-β regulatory element. Gelinas’ group has demonstrated c-Rel–dependent assembly of an enhanceosome-like complex in the promoter region of bfl-1/A1, which is required for induced expression (Edelstein et al). Our study suggests CD40 as a likely component of such an enhanceosome complex. Further investigation is needed to determine whether additional components are also involved in this transcriptional complex for activating cell growth and survival genes regulated by the NF-κB signaling pathway.

Along with the discovery of the nuclear expression of the plasma membrane–associated CD40 protein, downstream cytoplasmic components of CD40 signaling pathways, such as NF-κB family regulatory components—the IKKs (components of the CD40 signalosome)—have also been recently shown to enter the nucleus. In the nucleus, IKKα binds specific promoter regions in conjunction with the CREB-binding protein, resulting in phosphorylation of histone H3 and the promotion of transcriptional activity. TRAF6, another component of the CD40 signalosome, has also been shown to have a nuclear presence in lymphoma cells, indicating that components of the CD40 signalosome may translocate together or individually into the nucleus and possibly reassemble in various combinations to function as enhanceosomes or related types of macromolecular complexes to regulate the transcription of NF-κB target genes.

In addition to CD40 forming a signalosome on the plasma membrane of aggressive B-lymphoma cells, we have described in this study that CD40 can enter the nucleus of B cells and interact with c-Rel on the promoters of NF-κB target genes—CD40L, BLyS, and Bfl-1/A1—thereby further up-regulating the expression of these genes and enhancing cell proliferation. The potential targets of nuclear CD40 appear to correspond to the pathways activated by the CD40 plasma membrane counterpart, suggesting that blocking CD40-NF-κB pathway may involve not only signal transduction for cytoplasmic NF-κB activation, but also CD40 migration into the nucleus for full activation of NF-κB–regulated gene promoters. These findings may influence future therapeutic targeting strategies for aggressive B-cell lymphomas, such as LBCL.

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Authorship

Contribution: H.-J.Z. designed and performed experiments and wrote the paper; L.V.P. and Y.-C.L.-L., performed experiments; A.T.T., L.F., and L.C.Y. helped perform experiments; and R.J.F. supervised the project and wrote the paper.

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