Natural killer (NK) cells are innate lymphocytes that eliminate infected and transformed cells. They discriminate healthy from diseased tissue through killer cell Ig-like receptor (KIR) recognition of HLA class I ligands. Directly impacting NK cell function, KIR polymorphism associates with infection control and multiple autoimmune and pregnancy syndromes. Here we analyze KIR diversity of 241 individuals from five groups of Iranians. These five populations represent Baloch, Kurd, and Lur, together comprising 15% of the ethnically diverse Iranian population. We identified 159 KIR alleles, including 11 not previously characterized. We also identified 170 centromeric and 94 telomeric haplotypes, and 15 different KIR haplotypes carrying either a deletion or duplication encompassing one or more complete KIR genes. As expected, comparing our data with those representing major worldwide populations revealed the greatest similarity between Iranians and Europeans. Despite this similarity we observed higher frequencies of KIR3DL1*001 in Iran than any other population, and the highest frequency of HLA-B*51, a Bw4-containing allotype that acts as a strong educator of KIR3DL1*001+ NK cells. Compared to Europeans, the Iranians we studied also have a reduced frequency of 3DL1*004, which encodes an allotype that is not expressed at the NK cell surface. Concurrent with the resulting high frequency of strong viable interactions between inhibitory KIR and polymorphic HLA class I, the majority of KIR-A haplotypes characterized do not express a functional activating receptor. By contrast, the most frequent KIR-B haplotype in Iran expresses only one functional inhibitory KIR and the maximum number of activating KIR. This first complete, high-resolution, characterization of the KIR locus of Iranians will form a valuable reference for future clinical and population studies.

Keywords: NK cells, KIR, HLA class I, Iranian populations, immune diversity
INTRODUCTION

Natural killer (NK) cells are essential for human immunity to infection and cancer, and for successful reproduction (1, 2). To discriminate diseased from healthy tissue cells, NK cells express an array of inhibiting and activating cell surface receptors (3, 4). Prominent among these receptors are the killer cell immunoglobulin like receptors (KIR), which educate and modulate NK cell function through interaction with HLA class I (5, 6). KIR are highly polymorphic, a genetically-determined variation that directly impacts NK cell function, and susceptibility to disease (7, 8).

The KIR locus spans 150–350 kbp of chromosome 19q13.4 (9). The locus is distinguished by structural and sequence diversity of the 13 constituent genes (KIR2DL1, KIR2DL2/L3, KIR2DL4, KIR2DL5A, KIR2DL5B, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5, KIR3DL1/S1, KIR3DL2, and KIR3DL3) and two pseudogenes (KIR2DP1 and KIR3DP1) (10, 11). KIR have either two (2D) or three (3D) specificity-determining immunoglobulin-like domains and a long (L) or short (S) tail (12). KIR having a long cytoplasmic tail are inhibitory, whereas those having a short cytoplasmic tail are activating. The one exception is KIR2DL4, which can exhibit inhibitory or activating function (13–15). The KIR locus segregates in two main haplotype forms that are maintained in all human populations by balancing selection (16). The KIR-A haplotypes have a fixed number of predominantly inhibitory receptors, whereas the KIR-B haplotypes are characterized by a variable number of both activating and inhibitory receptors. Further distinguishing KIR-A and -B haplotypes are their characteristic alleles (17). KIR-A haplotypes are associated with controlling infectious disease and cancer, but confer susceptibility to reproductive disorders, whereas KIR-B haplotypes are associated with protection from reproductive disorders (1, 7). Additionally, haploidalent transplantation therapy for leukemia has an increased success rate when the stem cell donors carry KIR-B haplotypes (18, 19).

Polymorphism of KIR affects cell surface expression, ligand specificity, ligand binding strength, and intracellular signaling (20–25). All these factors affect the capacity of NK cells to recognize and kill target cells. Because both KIR polymorphism and associated diseases are unevenly distributed worldwide, it is critical to fully gauge the genetic diversity of KIR in well-defined human populations. Despite this importance, only a few populations have been studied to high resolution, principally due to the complexity of the KIR locus. These studies have focused on representative populations of Amerindians (Yucpa) (26), divergent African groups (27–29), Europeans (30, 31), East Asians (Japanese) (32), and Oceaneans (Māori) (33). Together, they show how KIR allele and haplotype diversity varies dramatically between human populations and highlight the importance of extending KIR allele analysis to represent all ethnicities and geographical areas. In this regard, our recent analysis of HLA allelic diversity in Iran revealed the highest frequencies of HLA-B*51:01 worldwide (34). HLA-B*51 contains the Bw4 epitope and interacts KIR3DL1 (35, 36). In the present study we fully characterize KIR locus diversity of the same cohort of Iranian individuals.

MATERIALS AND METHODS

Study Population

The KIR locus diversity of three indigenous Iranian populations was determined to high-resolution by analyzing genomic DNA from 241 healthy unrelated donors (34). The populations studied represent Baloch, Kurd, and Lur, together comprising 15% (12 million individuals) of the ethnically diverse Iranian population. Studied were 160 Lurs and 48 Kurds, from the Zagros Mountains at the west of Iran, and 33 Baloch from the southeast of Iran. The Lur population included 64 individuals from the city of Khoramabad in the province of Lorestan, 81 from Yasuj in the province of Kohgiluyeh and Boyer-Ahmad, and 15 from Lordegan in the province of Chaharmahal and Bakhtiari. The samples from Kurds were collected from the city of Sanandaj. With the exception of the Baloch, the HLA class I alleles were described previously (34). The ancestors of every individual studied had been part of their respective population for at least two generations. Sample collection was approved by the Medical Research Ethics Committee of Shiraz University of Medical Sciences. All participants gave informed consent. Banked, de-identified samples were used for this study.

Library Preparation and Enrichment

Genomic DNA was prepared by shearing with sonication and the KIR genomic region was enriched from the genomic libraries using a pool of oligonucleotide probes as described (37). The enriched fragments were subjected to paired-end sequencing using Illumina’s MiSeq instrument and V3 sequencing chemistry (Illumina, La Jolla CA). The sequencing read length was 2 × 300 bp.

Next Generation Sequence Data Processing and Analysis

Sequence reads specific to the KIR region were identified and harvested using Bowtie 2 (38). KIR genotyping was performed using the Pushing Immunogenetics to the Next Generation pipeline (37). This pipeline generates a high-resolution KIR gene content and allele level genotype. It can also identify previously unreported single nucleotide polymorphisms (SNPs) and recombinant alleles. Novel allele sequences were analyzed by visual inspection: reads specific to the relevant gene were isolated by bioinformatics filtering, aligned to the closest reference allele using MIRA 4.0.2 (39), and inspected using Gap4 of the Staden package (40) or Integrative Genomics Viewer (41).

Allele and Haplotype Frequencies

Allele frequencies were calculated by direct counting. The composition and frequencies of KIR haplotypes were determined at the allelic level using PHASE 2.1 (42). The following parameters for PHASE 2.1 were used: −f1, −x5, and −d1. Because of the high rate of recombination between KIR3DP1 and KIR2DL4 (9), we performed two separate PHASE runs, one for the KIR genes of the centromeric region and one for telomeric KIR genes. Genes analyzed were KIR3DL3, 2DS2, 2DL2/3, 2DL5A and B, 2DS3/5, 2DP1, 2DL1, 2DL4, 3DL1/S1,
FIGURE 1 | Iranian KIR genotypes resemble those of Europeans. (A) Shows KIR gene copy-number genotypes ordered by the total number observed across the five Iranian populations. Only genotypes present in more than one individual are shown. Colored boxes indicate the number of copies of the gene, as given in the key (Continued)
2DS1, 2DS4, and 3DL2. For each haplotype we calculated the frequency by direct counting.

**Statistical Analysis**

Hardy-Weinberg equilibrium proportions was examined using Fisher’s exact test. Differences in frequency amongst populations were tested using χ² with Bonferroni correction for the number of alleles at the respective locus. Fisher’s and χ² tests as well as Mann-Whitney U-test were implemented using GraphPad Prism 7.05.

**Genetic Distance**

The genetic distance between Iranians and other populations was calculated using the Cavalli-Sforza model (43) implemented in GENETIX 4.05 (https://kimura.univ-montp2.fr/genetix/). The populations used were Japanese (N = 115) (32), Yucpa Amerindians (N = 61) (26), Ghanaians from West Africa (N = 131) (27), Māori from New Zealand (N = 49) (33), Europeans (N = 378) (44), and Khomani from South Africa (N = 79) (28).

**RESULTS**

We sequenced the KIR genes of 241 individuals from five populations, representing three groups of Iranians; the Lurs, Kurds and Baloch. The Lurs comprised individuals from the cities of Khoramabad, Lordegan and Yasuj. Only two KIR genes were present as two copies (2N) in every individual, KIR3DL3 at the centromeric end of the KIR locus and KIR3DL2 at the telomeric end (Figure 1A). Every KIR haplotype in this Iranian cohort is therefore flanked by these two framework genes. The third framework gene is KIR2DL4 (9). Because eight individuals have only one copy of KIR2DL4 and six have three copies, KIR2DL4 is not present on every Iranian KIR haplotype (Figure 1A), but is likely duplicated on some haplotypes and deleted from others (45). The frequency of KIR-A haplotypes varies across populations (16, 46). In the combined study population, the frequency of KIR AA genotypes observed is 25% (Figure 1B). Centromeric KIR-A haplotypes are present at 65% and telomeric KIR-A haplotypes at 76% (Figure 1C). The frequency of KIR A haplotype homozygotes closely matches that of European populations (Figure 1B), as do genetic distance measurements calculated from the KIR genotype data (Figure 1D). In conclusion, the number and distribution of KIR gene content haplotypes in Iranians closely resemble those present in Europeans.

We determined the KIR allele frequencies in the five Iranian populations. These data are shown in Supplementary Material A and summarized in Figure 2. The allele frequencies were consistent with Hardy-Weinberg equilibrium. Among the five populations, we identified 115 inhibitory KIR alleles and 18 activating KIR alleles (Figure 2A). Also present are 12 KIR2DL4 alleles. Inhibitory KIR are highly polymorphic in Iranians, with 12 KIR2DL1, 9 KIR2DL2/L3, 10 KIR2DL5, 22 KIR3DL2, 18 KIR3DL1, and 44 KIR3DL3 alleles observed in total. As in other populations, the activating KIR are less polymorphic than the inhibitory KIR, with 7 KIR2DS4 alleles, 8 KIR2DS3/5 alleles, two KIR2DS1 and two KIR2DS2 alleles and one KIR3DS1 allele being seen (Figure 2A). In Iranians, KIR3DL3 is the most polymorphic KIR gene, whereas KIR2DS1 and KIR2DS2 are the least variable. In the combined population analyzed, the most frequent allele for each of the inhibitory KIR are 2DL1*00302, 2DL2*00101, 2DL3*00101, 3DL1*00101, 3DL2*00101, and 3DL3*00301, respectively (Supplementary Material A). The most common KIR2DL4 and KIR2DS4 alleles are 2DL4*00801 and 2DS4*003, respectively. For the other activating KIR (KIR2DS1-3 and 2DS5) as well as KIR2DL5, the most frequent allele observed is absence of the gene (Supplementary Material A). We did not observe any statistically significant difference in frequency of any specific KIR allele between Kurs and Lurs. We identified 11 novel KIR alleles, eight being defined by amino acid substitutions, one by a synonymous substitution and two by substitutions in KIR2DP1 pseudogene (Figure 2B). All these novel alleles were observed in one or two individuals (Figure 2B). Seven of them have sequences identical to ones reported recently in a survey of more than one million registered bone marrow donors (47).

On the basis of allele composition, we defined 170 centromeric and 94 telomeric KIR haplotypes (Supplementary Materials B,C). Thus, by distinct haplotype number, the centromeric region is twice as diverse as the telomeric region. Emphasizing this difference, the 55 most frequent centromeric haplotypes account for 75% of the total haplotypes, whereas only 13 telomeric haplotypes are sufficient to account for 75% of the telomeric haplotypes (Figures 3A,B). In conclusion, the centromeric KIR region of Iranian KIR haplotypes is far more diverse than the telomeric KIR region.

Although KIR-A haplotypes are more numerous and frequent than KIR-B haplotypes (Figure 1), the most frequent centromeric region and the second most frequent telomeric region haplotypes are KIR-B (Figures 3C,D). Together, these centromeric and telomeric segments encode only one inhibitory receptor specific for HLA class I (KIR2DL1*001). Also encoded are KIR2DL1*004, an attenuated receptor (23) and KIR3DL2*007, which has a mutation in the first ITIM of the cytoplasmic tail (48) and thus may not transmit an inhibitory signal. By contrast, the KIR-B haplotype encodes three functional activating receptors (KIR2DS1, 2DS2, and 3DS1) and a full-length KIR2DL4 (∗005). This combination of common centromeric and telomeric KIR-B
haplotypes can therefore provide the maximum number of activating KIR. The most frequent KIR-A haplotype encodes four inhibitory receptors specific for polymorphic HLA class I and carries KIR2DS4*003, which is not-expressed because of a 22 bp deletion in exon 5 (11). Indeed, the frequency of the KIR2DS4 22 bp-del variant in Iranians is 0.62, more than four times higher than the frequency of the full-length variant (Figure 3E). Consequently, almost all KIR-A haplotypes in Iranians encode four functional inhibitory receptors and no activating receptor specific for polymorphic HLA class I.

A characteristic of the KIR locus is the occurrence of large-scale duplication or deletion events that encompass complete genes, which synergizes with allele variation to enhance KIR functional diversity (45, 49, 50). In the Iranian cohort, we identified seven KIR haplotypes having large deletions and eight with duplications (Figure 4). One of these haplotypes (number 1 in Figure 4) was not observed previously and is similar to the most frequent KIR-B haplotype in Iran, with the difference being that it lacks KIR2DS1. This haplotype could have been formed by homologous or looping out recombination (50). The remaining
FIGURE 3 | Centromeric KIR are more diverse than telomeric KIR in Iranians. (A) Shows the number of distinct haplotypes observed in the centromeric and telomeric KIR regions. (B) Shows the cumulative frequency of haplotypes observed in the centromeric and telomeric KIR regions. (C,D) Shows the allele composition of all centromeric, and (D) telomeric KIR haplotypes identified in more than ten individuals in the combined Iranian population. KIR A haplotypes are shaded pink and KIR B haplotypes are blue. Empty boxes indicate gene absence. At the right is shown number observed and the frequency in the combined Iranian population (N = 241). All the observed haplotypes are given in Supplementary Material (E). Shown are the frequencies of KIR2DL4 and KIR2DS4 alleles in the combined Iranian population. Red text indicates alleles not expressed at cell surface. The allele frequencies for all KIR genes in each of the five populations are given in Supplementary Material.
deletion haplotypes are similar to those observed worldwide including Africans, and the duplication haplotypes similar to those observed outside of Africa (45).

The interaction of HLA class I with inhibitory KIR contributes to NK cell education (51, 52). We analyzed the distribution of alleles for the four inhibitory KIR that are specific for HLA class I in Iranians and compared them with six other populations that represent the breadth of human genetic diversity (Figure 5). This analysis showed that in Iranians, Europeans, West Africans, and Japanese the most frequent KIR2DL1 and KIR2DL2/3 alleles are 2DL1*003 and 2DL3*001, respectively. The two populations that differ are relatively small indigenous populations (Figures 5A,B). By contrast, the KIR3DL1/S1 and KIR3DL2 alleles most frequent in Iranians are usually not the same as those most frequent in other populations (Figures 5C,D). This shows there is a greater worldwide divergence of KIR specific for HLA-A and -B than of KIR specific for HLA-C. Of particular note, Iranian populations have the highest frequency of 3DL1*001 (0.29) compared to the other six populations as well as to all other populations analyzed to date (53). Allele frequencies of the four inhibitory KIR specific for HLA class I are similar across the Iranian populations analyzed (Figures 5E–H). The one exception is the Baloch who have a low frequency of KIR3DL1*004, as well as KIR3DL2*003 and *005, which are in strong linkage disequilibrium with 3DL1*004 (28, 44) (Supplementary Material C). The Baloch have one tenth the frequency of 3DL1*004 (0.015) than Kurd [0.114, χ² p = 0.006; pc = 0.06(ns)], Lorestan [0.109, χ² p = 0.006; pc = 0.06(ns)] and Yasuj [0.129, χ² 2 p = 0.003; pc = 0.03]. KIR3DL1*004 is retained in the cytoplasm and unable to bind HLA-Bw4+ HLA-A or -B on target cells (54).

We examined the compound genotypes of KIR and HLA class I, to determine the potential number of interactions between HLA class I ligands and inhibitory KIR. We observed a consistent mean of four viable interactions per individual of HLA-C with inhibitory KIR across the five Iranian populations (Figure 6A). This number is similar to the mean of 3.6 observed in Europeans (28). Although we observed no significant difference in the potential interactions of KIR3DL1 with HLA-A, the Baloch and Lordegan have more interactions of KIR3DL1 with HLA-B than the other three Iranian populations (Figures 6B,C). Despite the small numbers of individuals in these groups, the differences are statistically significant (Mann-Whitney U-test; p < 0.013). Contributing to these differences is the high frequency of HLA-B*51 in the Baloch (0.29; Supplementary Material D). This frequency is similar to the 0.28 observed in the Lordegan and considered the highest worldwide (34).

**DISCUSSION**

This study applied high-throughput, next-generation sequencing to define KIR polymorphism at high resolution in five Iranian populations. These comprise the Kurd and Lur populations from the Zagros Mountains in the west of Iran and the Baloch from the south-east. The Lur comprised three subpopulations; the Lorestan, Lordegan, and Yasuj. HLA class I allele distributions for four of these populations were reported previously (34) whereas those for the Baloch are described here. When we compared the KIR allele frequencies of Iranians to those representing African, Asian, European, Oceanian, and South American populations, we found that the Iranian groups we studied are particularly similar to Europeans. This finding is consistent with ancient DNA analysis, which revealed that Iranians and Europeans both originate from an Indo-Europeans steppe ancestor population (55).

Despite their overall similarity, the Iranians we studied differ from Europeans in their frequencies of KIR3DL1*001, a high-expressing inhibitory receptor specific for the Bw4 epitope of
FIGURE 5 | Telomeric KIR are more divergent than centromeric KIR across populations. Shows the alleles of (A) KIR2DL1, (B) KIR2DL2/3, (C) KIR3DL1/S1, and (D) KIR3DL2 observed in Iran, and their frequencies in the combined Iranian population and six other populations representing major world groups (Left). (E-H) show the allele frequencies in the individual Iranian populations. The populations are Iranians (N = 241, comprised from Baloch N = 33, Kurds N = 48, Lorestan N = 64, Lordegan N = 15, and Yasuj N = 81), Ghanaians (N = 131), Khomani (N = 79), Maori (N = 49), Japanese (N = 115), Europeans (N = 378), and Yucpa (N = 61). The allele frequencies for all KIR genes in each of the five Iranian populations are given in Supplementary Material.
subsets of HLA-A and -B allotypes. Iranians have KIR3DL1∗001 frequencies that are twice those in Europeans and are the highest worldwide. Iran has the highest frequency of HLA-B∗51 in the world (34), and this is also the case for the Baloch. HLA-B∗51 has the Bw4 epitope and thus educates KIR3DL1∗ NK cells to detect any loss in Bw4∗ HLA-A or -B expression (35, 56, 57). HLA-B∗51 is associated with Behçet disease, a chronic, multi-system autoimmune condition that has substantially higher incidence in Iran (80/100,000) than Europe (<1/100,000) (58, 59). We recently showed that high-expressing allotypes of KIR3DL1, including 3DL1∗001, can protect from Behçet disease (60). It is unlikely that KIR3DL1∗001 and HLA-B∗51 rose to high frequency in Iran to protect specifically from an autoimmune disease, but this combination of HLA and KIR could also protect against specific infectious diseases (7). Examples include tuberculosis and hepatitis, which are both prevalent in Balochistan (61–63). Amongst Iranians, the Baloch and Lordegan have the highest number of viable interactions between KIR3DL1 and Bw4∗HLA. Contributing to this high occurrence in the Baloch, are high frequencies of KIR3DL1∗001 and HLA-B∗51 and a low frequency of KIR3DL1∗004. That both the KIR3DL2 alleles linked to KIR3DL1∗004 are also reduced in frequency suggests that KIR3DL1∗004 has been specifically targeted by negative selection in the Baloch, rather than another KIR allele in linkage disequilibrium with KIR3DL1∗004.

KIR-A and -B haplotypes are present in all human populations, where they are maintained by balancing selection, likely because KIR-A haplotypes favor infection control, particularly viral infections, and KIR-B haplotypes favor successful fetal implantation (1, 16). Accordingly, KIR-A haplotypes express all possible inhibitory receptors specific for HLA class I, whereas KIR-B haplotypes express fewer inhibitory receptors but more activating receptors. Thus, whereas the inhibitory KIR help prime NK cells to be able to be responsive to HLA class I loss during infection, the activating KIR can both promote fetal trophoblast invasion and recognize specific pathogen-derived peptides to control certain infections (64, 65). In Iranian populations the differences between the KIR-A and KIR-B haplotypes is extreme. The common KIR-A haplotypes express no activating KIR, due to the high frequency of the truncated KIR2DS4 variant, and the common KIR-B haplotypes provide the maximum number of activating receptors. In this regard, the KIR-B haplotypes differ considerably from those of Africans and were likely obtained since the out of Africa migration, through adaptive introgression with ancient humans (66).

In summary, we describe the KIR locus at allelic resolution in Iranian populations and place it in the context of the HLA ligands recognized by KIR. Iran is a culturally diverse country and the ethnic groups we have studied comprise ~15% of the population (67). Because substantial KIR gene content diversity is observed across Iran (68–71), it will be of interest in future studies to compare our results with other Iranian populations including Persians and Azeris. The allele and haplotype distributions described here will provide a baseline for future studies of disease association and transplantation matching in this important region of the world.

DATA AVAILABILITY STATEMENT
All relevant data are included in the article/Supplementary Material. Other raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT
The studies involving human participants were reviewed and approved by the Medical Research Ethics Committee of Shiraz University of Medical Sciences. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS
PP, EA, AG, and PN conceived and designed experiments. EA, NN-G, and ST performed lab experiments. CA, PN, JAT, LG, NN-G, and ST analyzed data. AG, PP, WM, JH, JT, and LM provided materials. CA, PN, and PP wrote the paper. All authors approved the final submitted version.
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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00556/full#supplementary-material

Supplementary Material (Spreadsheet) (A) Shows the allele frequencies for the 13 KIR genes in five Iranian populations, and the combined frequency is shown at the right. (B) Shows are the centromeric KIR haplotypes identified in five Iranian populations. The combined frequency is shown at the right. KIR A haplotypes are shaded in red, KIR B haplotypes are shaded in blue. (C) Shows are the telomeric KIR haplotypes identified in five Iranian populations. The combined frequency is shown at the right. KIR A haplotypes are shaded in red, KIR B haplotypes are shaded in blue. Yellow shading indicates novel KIR alleles identified in this study, which are described in Figure 2. (D) Shows the HLA class I allele frequencies observed in the Baloch population.
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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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